Bovine Apolipoprotein E in Plasma: Increase of ApoE Concentration Induced by Fasting and Distribution in Lipoprotein Fractions

Yuji TAKAHASHI1), Kan SATO2), Fumiaki ITOH3), Toru MIYAMOTO1), Tsutai OOHASHI1) and Norio KATOH4)

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ABSTRACT. Apolipoprotein E (apoE) is a protein constituent of lipoproteins, and acts as a receptor-binding ligand. Although the existence of bovine apoE in lipoprotein fractions has already been reported, quantitative studies on the changes of apoE in plasma and lipoprotein fractions are lacking. In the present study, an increase of a 38 kDa protein in the very low-density lipoprotein (VLDL) fraction obtained from fasted calves was detected. This 38 kDa protein was identified as bovine apoE by determination of the N-terminal amino acid sequence. Bovine apoE was purified and an enzyme-linked immunosorbent assay (ELISA) was developed. Using this system, the effect of fasting on the concentration of apoE in plasma and the distribution of apoE in lipoprotein fractions were investigated. After 3 days of fasting, the concentration of plasma apoE increased significantly (p<0.05) by 280 %, and was returned to the basal level by 3 days of refeeding. The lipoprotein fractions obtained from before and after fasting was separated by ultracentrifugation. ApoE was significantly increased in VLDL, low-density lipoprotein (LDL) and non-lipoprotein fractions by fasting (p<0.05). On the other hand, in high-density lipoprotein (HDL) fractions obtained from both before and after fasting, the level of apoE was very low compared to the other fractions. These results suggested that bovine apoE contents in triglyceride-rich lipoproteins are modulated by nutritional treatment and closely associated with triglyceride-rich lipoprotein metabolism.

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molecular masses of proteins.

Determination of N-terminal amino acid sequence: The 38 kDa protein found in the VLDL fraction from day 3 fasting calves was isolated by SDS-PAGE, and was transferred to a polyvinylidene difluoride (PVDF) 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 38 kDa protein bands were cut from membrane, and sequenced on a protein sequencer (Model 491, Applied Biosystems Japan Ltd., Tokyo, Japan) using standard procedures.

Purification of apoE: ApoE was isolated from bovine plasma according to the method by Avila et al. [2] with slight modification. Five Holstein calves were fasted for 3 days. Blood samples of about 400 ml containing 1.0 mg/ml EDTA were taken from each calf after 3 days of fasting. The VLDL plus chylomicron fractions were prepared by ultracentrifugation (d<1.021 g/ml) and were delipidated with 5 volumes of acetone/ethanol (1:1, v/v) at 4°C. The precipitated protein was isolated by centrifugation. To ensure complete delipidation, the extraction procedure was repeated three times. Finally, the precipitate was washed at 0°C with 5 volumes of acetone/ethanol (1:1, v/v) at 4°C. EDTA were taken from each calf after 3 days. Blood samples of about 400 ml containing 1.0 mg/ml EDTA were taken from each calf after 3 days of fasting. The VLDL plus chylomicron fractions were prepared by ultracentrifugation (d<1.021 g/ml) and were delipidated with 5 volumes of acetone/ethanol (1:1, v/v) at 4°C. The precipitated protein was isolated by centrifugation. To ensure complete delipidation, the extraction procedure was repeated three times. Finally, the precipitate was washed at 0°C with 5 volumes of acetone/ethanol (1:1, v/v) at 4°C.

Preparation of anti-bovine apoE antiserum: Purified bovine apoE (0.3 mg) was emulsified in an equal volume of Freund’s complete adjuvant, and injected into rabbits. After 17 days, 0.2 mg of 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. Specificity of obtained antiserum was estimated by immunoblot analysis. Plasma and bovine apoE were electrophoresed, and gels were stained with Coomassie brilliant blue or were immunoblotted using anti-bovine apoE antiserum, and 5 to 20% gradient gels were used. After electrophoresis, plasma and purified bovine apoE in gel were transferred by immersing the membrane in 5% nonfat dry milk in Tris-buffered saline-Tween (20 mM Tris, 0.5 M NaCl, 0.05% Tween 20 [pH 7.5]; TBS-Tween) for 1 hr at room temperature on an orbital shaker. The membrane was washed 3 times with TBS-Tween, and incubated with anti-bovine apoE solution overnight at 4°C. Anti-bovine apoE antiserum was diluted 1:5,000 with TBS-Tween containing 5% nonfat dry milk. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Boehringer Mannheim GmbH, Mannheim, Germany) diluted 1:5,000 with TBS-Tween containing 5% nonfat dry milk for 1 hr at room temperature. Bovine apoE was detected by chemiluminescence (ECL kit, Amersham Biosciences).

Enzyme linked immunosorbent assay: Ninety-six-well polystyrene plates (Sumitomo Bakelite Co., Tokyo, Japan) were coated with non-labeling anti-bovine apoE IgG (1.0 µg per well) dissolved in 50 mM sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were inverted to remove the antibody solution, and residual binding sites were blocked for 1 hr at room temperature with PBS containing 2% bovine serum albumin (Sigma Chemical Co., St. Louis, U.S.A.). Wells were washed 6 times with PBS containing 0.05% Tween 20 (PBS-Tween). Samples diluted appropriately in PBS were applied to plates in duplicate, and incubated for 1 hr at room temperature. After 6 washes with PBS-Tween, biotin-labeling anti-bovine apoE IgG (0.1 µl), which had been diluted 1:200 with PBS-Tween, was added to each well. Plates were incubated for 1 hr at room temperature. After washing, alkaline phosphatase-conjugated streptavidin (Boehringer Mannheim GmbH) diluted 1:5,000 with PBS-Tween was added to each well (0.1 µl), and plates were incubated for 1 hr at room temperature. After washing, color was developed for 90 min by using the ELISA substrate system (alkaline phosphatase substrate kit, Bio-Rad Laboratories). Color development was measured at 405 nm with a microplate reader (Model 550, Bio-Rad Laboratories).

A standard curve was prepared for each assay by appropriate dilution of the standard plasma, which had been calibrated. The apoE concentration in the standard plasma was determined by densitometric analysis using SDS-PAGE following Coomassie brilliant blue staining. The standard bovine plasma (10 µg of apoE/ml) was serially diluted (10- to 2,560-fold dilution; 1.0 to 0.004 µg/ml). Standard curves were prepared by plotting absorbance at 405 nm as a function of the apoE concentration. A sigmoid curve was fitted to the data, and absorbance values were converted to con-
centrations.

Other methods: The concentrations of triglyceride, cholesterol, phospholipid and free fatty acid in plasma and lipoprotein fractions were measured using kits (Wako Pure Chemicals, Osaka, Japan). Protein concentration was determined by the method of Bradford [4] using bovine plasma gamma globulin (Bio-Rad Laboratories) as the standard.

Analysis of data: Data were analyzed by one-way ANOVA and Student’s t-test (unpaired).

RESULTS

Detection of 38 kDa protein in lipoprotein fractions separated by ultracentrifugation: Some series of sera obtained from calves starved for 3 days and refed for 3 days, were used for detection of apoE-like protein. Apolipoproteins in lipoprotein fractions were separated by SDS-PAGE and detected by Coomassie brilliant blue staining. A typical result is shown in Fig. 1. A slight protein band, whose molecular weight was estimated to be 38 kDa, was found in the VLDL fraction at day 0. The magnitude of this band gradually increased during the fasting period and returned to the basal level after refeding for 3 days. In the LDL fraction, similar 38 kDa bands were also detected; however, the changes due to fasting and refeeding were unclear (data not shown). No protein band corresponding to the 38 kDa protein was detected in any HDL sample obtained during the experimental period (data not shown). The N-terminal amino acid sequence of the 38 kDa protein found in the VLDL fraction was determined. The obtained sequence (D-M-E-G-E-L-G-P-E-E-P-L-T-T-Q-Q-P-R-G-K) was coincident with that deduced from the bovine liver apoE mRNA sequence [6]; therefore, the 38 kDa protein was identified as bovine apoE.

Purification bovine apoE and preparation of anti-bovine apoE antiserum: Bovine apoE was isolated from plasma using ultracentrifugation, heparin affinity and gel filtration. The purity of apoE was confirmed by SDS-PAGE and Coomassie brilliant blue staining. Only one band was stained (Fig. 2, lane 2). The specificity of the anti-bovine apoE antiserum was confirmed by SDS-PAGE and immunoblotting (Fig. 2, lanes 1, 3).

Enzyme-linked immunosorbent assay of bovine apoE: The standard curve made from a series of standard plasma dilutions is shown a typical sigmoid curve (Fig. 3). The intraassay coefficient of variance was in the range from 3.0 to 4.8% (n=15). The interassay coefficient of variance was 12.6% (n=5). The recovery of added apoE was in the range from 80 to 97%.

Changes of lipids in plasma and lipoprotein fractions separated by ultracentrifugation: The lipid concentrations in plasma and lipoprotein fractions before and after 3 days of fasting are summarized in Table 1. Total cholesterol (TC) and free fatty acid (FFA) concentrations in plasma significantly increased after fasting (p<0.01), but triglyceride and phospholipid (PL) concentrations did not change. In the VLDL fraction, the concentration of PL significantly increased 1.2-fold after fasting (p<0.01), but TC and TG were not changed. In the LDL fraction, TG decreased 36% compared with after feeding (p<0.01), whereas TC and PL concentrations significantly increased after fasting (p<0.01, TC: 1.3-fold, PL: 1.2-fold). In the HDL fraction, none of the lipid levels measured were changed by fasting.

Changes of plasma concentration of apoE induced by fasting and refeeding: The changes of plasma apoE during 3 days of fasting and following 3 days of refeeding are shown in Fig. 4. At day 0, the concentration of plasma apoE was 6.90 ± 1.08 µg/ml (n=4, mean ± SD). The plasma apoE concentration gradually increased during the fasting period, and reached 19.28 ± 3.16 µg/ml at day 3, which was 2.8-fold higher than that of day 0 (p<0.05). After calves were refed for 3 days (day 6), the concentration of plasma apoE returned to the initial level (7.10 ± 1.06 µg/ml).

Distribution of apoE among lipoprotein fractions separated by ultracentrifugation: The concentrations of apoE in lipoprotein fractions at day 0 (before fasting) and day 3 (after fasting) are summarized in Fig. 5. At day 0, the concentrations of apoE in VLDL, LDL, HDL and non-lipoprotein fractions were 2.33 ± 0.49, 1.68 ± 0.34, 0.28 ± 0.05, and 0.20 ± 0.04 µg/ml, respectively. After fasting, the concentration of apoE in the VLDL fraction significantly increased 2.8-fold (p<0.01), whereas the concentrations in the LDL and HDL fractions did not change (p>0.05). In the non-lipoprotein fraction, the concentration of apoE significantly increased 1.5-fold (p<0.01).
tein (d>1.21 g/ml) fractions were 0.86 ± 0.34, 2.98 ± 1.19, 0.16 ± 0.04, 2.55 ± 0.56 µg/ml (n=10, mean ± SD), respectively, and at day 3, 1.78 ± 0.74, 5.20 ± 2.90, 0.16 ± 0.07, 3.40 ± 1.65 µg/ml, respectively. Significant increases of apoE concentrations occurred in the VLDL (p<0.002), LDL (p<0.029) and non-lipoprotein (p<0.001) fractions. The magnitude of increase in apoE caused by fasting was larger in the non-lipoprotein fraction (3.8-fold) than in the VLDL (2.1-fold) and LDL (1.8-fold) fractions. The percentages of apoE in lipoprotein fractions were as follows: before fasting in VLDL, LDL, HDL and non-lipoprotein fractions they were 12, 38, 2, 35%, and after fasting, 10, 27, 1, 50%, respectively.

### DISCUSSION

It has been reported that apoE is found in several classes of bovine plasma lipoproteins; however, SDS-PAGE and protein staining were used for detection of apoE in these reports. In the present study, we found that the 38 kDa pro-

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**Table 1. Concentrations of Triglyceride, Total Cholesterol, Phospholipid and Free Fatty Acid in Plasma and Lipoprotein Fractions from before and after Fasting (3 days)**

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triglyceride (mg/dl)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>before fasted</td>
<td>21 ± 2</td>
<td>4.1 ± 0.5</td>
<td>5.8 ± 0.3</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>after fasted</td>
<td>20 ± 2</td>
<td>4.2 ± 0.5</td>
<td>3.7 ± 0.3*</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td><strong>Total Cholesterol (mg/dl)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before fasted</td>
<td>80 ± 4</td>
<td>1.05 ± 0.09</td>
<td>11.8 ± 0.9</td>
<td>45.9 ± 2.9</td>
</tr>
<tr>
<td>after fasted</td>
<td>97 ± 5*</td>
<td>1.12 ± 0.07</td>
<td>15.5 ± 0.7*</td>
<td>44.2 ± 4.0</td>
</tr>
<tr>
<td><strong>Phospholipid (mg/dl)</strong></td>
<td></td>
<td></td>
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<tr>
<td>before fasted</td>
<td>104 ± 5</td>
<td>0.161 ± 0.008</td>
<td>9.9 ± 1.0</td>
<td>44.4 ± 4.1</td>
</tr>
<tr>
<td>after fasted</td>
<td>107 ± 5</td>
<td>0.195 ± 0.007*</td>
<td>12.1 ± 0.9*</td>
<td>51.4 ± 2.8</td>
</tr>
<tr>
<td><strong>Free Fatty Acid (mEq/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before fasted</td>
<td>0.16 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>after fasted</td>
<td>0.84 ± 0.04*</td>
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</table>

Each values is the mean of 10 calves ± SD. * P<0.05, compared with the value before fasting.
protein increased in the VLDL fraction obtained from fasted calves, and determined the N-terminal amino acid sequence of this protein. The resulting sequence was coincident with that of apoE found in bovine liver [6], cerebrospinal fluid [16] and corpus luteum [23]. The 38 kDa protein was, therefore, identified as bovine apoE.

The concentration of apoE in bovine plasma was in the range from 4.2 to 27.4 µg/ml as estimated by ELISA in the present study. These values were low compared with those reported in other species. In a normal healthy individual, serum levels of apoE were reported to be about 30–70 µg/ml [26] in the human, and 150–320 µg/ml in the rat [25]. The low concentration of bovine plasma apoE might explain the reason why several authors could not find any apoE-like protein in bovine lipoprotein fractions [3, 5, 19].

The concentration of total apoE in plasma was significantly increased by fasting for 3 days, and was returned to the initial level by refeeding for 3 days (Fig. 4). This result was similar to that reported for rats. Davis et al. reported that the serum of fasted rats contained 62% more immunoreactive apoE than that of the control [9]. In cultured hepatocytes from fasted rats, apoE mRNA and apoE secretion increased by 2-fold and 2.3-fold, respectively [10]. In calves, fasting seemed to stimulate apoE synthesis and secretion by the liver, as in the rat.

The concentration of apoE in the VLDL fraction was increased 2.1-fold by fasting for 3 days (Fig. 5). This result was similar to those of previous studies using fasting rats and cultured hepatocytes. Oleate increased incorporation of [3H] leucine into VLDL apolipoprotein in fasted rats, and the increased incorporation of [3H] leucine was mainly into VLDL-apoE [27, 28]. In the human hepatoma cell line HepG2, enhanced association of apoE with large, apoB-containing lipoproteins occurs when cellular lipogenesis and VLDL production are stimulated [12]. In the present study, an increase of VLDL-apoE occurred concomitant with marked elevation of plasma FFA in fasted calves (Table 1). After being taken up by the liver, FFA is reesterified to TG, and then secreted as VLDL particles. The increase of VLDL-apoE, therefore, seemed to be attributable to the increase of VLDL secretion. Although VLDL-TG was unaffected by fasting, this was presumably due to rapid turnover rate of TG under fasting conditions, compared to that of apolipoproteins.

The concentration of apoE in the LDL fraction significantly increased after 3 days of fasting (Fig. 5). This might have been the result of an increase in intermediate-density lipoproteins (IDL), as well as enhancement of VLDL secretion from the liver. VLDL-TG is hydrolyzed by lipoprotein lipase (LPL) in the plasma, and small, apoE-enriched and cholesterol-enriched lipoproteins known as IDL are generated [22]. Since the density range of IDL is 1.006–1.019 g/ml, IDL was separated in the LDL fraction in the conditions used in the present study. The concentration of TG was significantly decreased in the LDL fraction, whereas those of cholesterol and phospholipids were significantly increased by fasting (Table 1). The changes of lipid composition in the LDL fraction might reflect the increase of IDL particles in this fraction.

The concentration of apoE in the HDL fraction was low compared with those of VLDL or LDL, and was not changed significantly by fasting (Fig. 5). Most previous studies did not find apoE in bovine HDL, even if it was found in VLDL or LDL [13, 24]. Brantmeier et al. showed the absence of apoE in HDL by separation of bovine HDL using Heparin-Sepharose affinity chromatography [5]. In the human, however, HDL is one of the major sources of

**Fig. 4.** Changes of plasma apoE concentrations caused by fasting and refeeding. Calves were fasted for 3 days followed by refeeding for 3 days. Blood samples were taken before fasting (day 0), after fasting (days 1, 2, and 3), and after 3 days of refeeding (day 6). The concentrations of apoE in plasma were measured by ELISA. Values are expressed as mean ± SD (n=10). Values with different letters are significantly different at P<0.05.

**Fig. 5.** Effects of fasting on the apoE concentrations in lipoprotein fractions. Blood samples were taken before (day 0), and after (day 3) fasting. Lipoprotein fractions were prepared by ultracentrifugation. The concentrations of apoE in plasma were measured by ELISA. Values are expressed as mean ± SD (n=10). * P<0.05, compared with the value before fasting (day 0).
apoE. HDL-apoE is believed to have an important role in cholesterol metabolism, mediating hepatic uptake of HDL, which would be the final phase of the hypothesized reverse transport of cholesterol [22]. In ultracentrifugal separation of the lipoproteins, the percentage of apoE in HDL was 25% in the human [20]. This value was markedly larger than those of our results, in which only 2% (before fasting) and 1% (after fasting) of total plasma apoE were distributed in the HDL fraction. Therefore, it is considered that there is less apoE in the bovine HDL fraction than in other species.

A large amount of plasma apoE was found in the non-lipoprotein fractions separated from the sera both before (35%) and after fasting (50%). Ultracentrifugation of human plasma resulted in up to 40% of apoE appearing in the non-lipoprotein fraction [20]. When rat sera are centrifuged at a density of 1.21 g/ml, marked quantities of apoE (11–22%) are recovered in the non-lipoprotein fractions [8]. Although the origin of apoE in non-lipoprotein fractions is unclear, there are two possibilities. One is the result of dissociation from lipoprotein particles. Ultracentrifugal procedures are conditions of high salt and high centrifugal force, and strip the protein from the lipoprotein particles [20]. Another is that bovine apoE is secreted in a lipid-deficient form. In isolated rat hepatocytes, the majority of the apoE secreted from cells was eluted in fractions that contained no detectable lipid [10]. Since it is known that apoE can move among several classes of lipoproteins, lipid-free apoE may provide additional apolipoprotein available for association with the TG-rich lipoproteins [12], where apoE has a role in metabolism of these lipoproteins as a ligand of remnant receptors.

Increased concentrations of plasma apoE in the fasting cow imply the possible relevance of apoE in the development of lipid-related disorders such as fatty liver in the peripartum period. It is tempting to assume that detection of an unusually low apoE concentration in the peripartum period may be useful for early diagnosis of such disorders.

In conclusion, we found an increase of a 38 kDa protein in bovine VLDL fraction caused by fasting, and the protein was identified as bovine apoE by determination of its N-terminal amino acid sequence. The concentration of bovine plasma apoE was very low compared with other species. ApoE was markedly increased in plasma and VLDL, LDL and non-lipoprotein fractions by fasting. In HDL fractions obtained from both before and after fasting, however, apoE had a very low value compared with those of VLDL and LDL. These results suggested that bovine apoE contents in TG-rich lipoproteins are modulated by nutritional treatment and closely associated with the TG-rich lipoprotein metabolism.

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