Decreases in Serum Apolipoprotein C-III Concentration in Cows with Ethionine-Induced Fatty Liver

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ABSTRACT. To monitor the serum concentration of apolipoprotein C-III (apoC-III), one of the functional apoproteins in lipid metabolism, in cows with ethionine-induced fatty liver, and to investigate the association of apoC-III with liver triglyceride (TG) content and serum biochemical variables, seven nonpregnant nonlactating Holstein cows (3 to 6 years old) were used. Five cows were treated with ethionine, an analogue of methionine, (days 0, 7 and 14). The remaining two controls received saline as the vehicle. Liver TG contents in the treated cows were increased markedly whenever administered, and significant increases were observed at days 14 (666.4%, 85.3 mg/g) and 21 (675.0%, 86.4 mg/g) compared with day 0. In controls, no significant changes in liver TG content and serum biochemical variables were observed during this experiment. The serum apoC-III concentration in the treated cows was decreased drastically after the first administration and fell to the lowest value at day 10 (76.2 µg/ml, 32% of day 0). The apoC-III was significantly (p<0.05) correlated with non-esterified fatty acids (r=-0.526), γ-glutamyl transpeptidase (r=-0.407), total bilirubin (r=-0.464), positively with apolipoprotein B-100 (apoB-100, r=0.601) and cholesterol ester (r=0.449). Although apoB-100 concentrations were also reduced by the administrations, the concentrations tended to recover smoothly toward the next administration. The distinct difference in change between apoC-III and apoB-100 suggests that apoC-III may be regulated by other pathways, in addition to inhibiting the synthesis of apoproteins by ethionine.

KEY WORDS: apolipoprotein C-III, bovine, ethionine, fatty liver.

During the final days prepartum and the first month of lactation, dairy cows are easily fall into negative energy balance caused by reduced feed intake, stress at calving, the start of milk lactation or a combination of these factors [12]. The energy imbalance mobilizes large amounts of non-esterified fatty acids (NEFA) from adipose tissue to the liver. When the excessive influx exceeds the metabolic capacity of the liver, the accumulation of triglyceride (TG) is increased and results in the development of hepatic lipodosis [12, 13]. This pathogenesis is known as the most common background of periparturient disorders in dairy cattle.

Apolipoprotein C-III (apoC-III) is recognized as one of the most important serum TG regulatory factors [8]. Bovine apoC-III is a low molecular mass protein mainly distributed in the high-density lipoprotein (HDL) fraction [7, 22] and consists of at least two isoforms, 8.2 and 7.3 kDa; the latter is the major species [23]. ApoC-III inhibits the activation of lipoprotein lipase by apolipoprotein C-II [1] and further reduces hepatic uptake of the remnant lipoprotein [19]. Lechthin:cholesterol acyltransferase (LCAT) is also activated by apoC-III [6, 15]. In mice, overexpression of the apoC-III gene results in hypertriglyceridemia [5], whereas disruption of the apoC-III gene induces hypotriglyceridemia [10].

The bovine serum apoC-III concentration has the lowest value in the nonlactating period, gradually increases around the early lactating stage and reaches the maximum concentration in the midlactation period [21]. Although studying apoC-III seems to be important to understand periparturient disorders in dairy cattle, only a few studies on the clinicopathological aspects of the relationship between apoC-III and periparturient diseases have been reported. The serum apoC-III concentration was reported to be increased in a calf with hyperlipidemia [23], whereas it was decreased in cows with fatty liver, ketosis, left displacement of the abomasum, milk fever and retained placenta, compared with healthy cows in the early lactation stage [24]. It was remarkably decreased in cows with milk fever [24]. Moreover, in milk fever, the serum apoC-III concentration is decreased several days before typical clinical signs appear [9]. These data suggest that a reduced apoC-III concentration is related to the pathogenesis of fatty liver and apoC-III can be a susceptible marker for prevention of periparturient diseases in clinical field. However, the change of apoC-III in the development of fatty liver has not yet been investigated. Therefore, we especially focused on the association of fatty liver with the serum apoC-III concentration in this study. The administration of ethionine was adopted as a method for induction of fatty liver in the cow because this experiment has been reported as a model which can mimic natural cases of fatty liver in relation to decreased concentrations of apoproteins [17]. Moreover, an apoC-III quantitative method...
was developed for a more sensitive sandwich enzyme-linked immunosorbent assay (ELISA) system.

The purpose of the present study was to monitor the serum apoC-III concentration in the development of experimentally induced fatty liver as well as its relevance to other lipid-related components.

MATERIALS AND METHODS

Cows: Seven clinically healthy Holstein cows (3 to 6 years old, 544 to 670 kg) were used. All cows were non-pregnant and nonlactating. They were handled under the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which are basically in conformity with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the U.S.A. (NIH publication No. 86-23, revised 1985).

DL-ethionine administration: Ethionine administration was carried out in accord with Uchida et al. [17]. The agent was injected intraperitoneally into five cows at a dose of 25 mg/kg of body weight under sedation with xylazine (0.07 mg/kg body weight, intravenously). The administrations were carried out 3 times (days 0, 7 and 14, Fig. 1). The remaining two cows used as controls were administered saline as the vehicle at the same intervals.

Serum and liver specimens: Blood samples were collected from the jugular vein every other day in the morning before feeding (0 to 28 days). Sera were prepared in an aseptic manner. Liver biopsy was performed at 0, 7, 14 and 21 days after the first administration. Briefly, to obtain adequate restraint, the cows were lightly sedated with xylazine as mentioned above and the biopsy area was infiltrated with 10 ml of 2% procaine hydrochloride. Liver samples were taken from the 7th to 12th intercostal space according to an ultrasonographic image (Model EUB-26, Hitachi Co., Japan). Samples were washed immediately with phosphate-buffered saline (PBS), and then frozen quickly in liquid nitrogen. All specimens were stored at –80°C until analysis.

Sandwich enzyme-linked immunosorbent assay for apoC-III: The sandwich ELISA method established was a modification of a previous method [21]. Briefly, purified rabbit anti-bovine-apoC-III antibody was diluted with 0.05 M carbonate buffer (pH 9.6), and pipetted into each well of a 96-well flat-bottom ELISA plate (Corning Inc., U.S.A.). The microplate was sealed and incubated overnight at 4°C. After the overnight period, the antibody solution was aspirated and each well was washed 6 times with 0.05% Tween 20 containing PBS solution (T20-PBS). To block any unbound site, 1.5% gelatin containing PBS solution (G-PBS) was dispensed into each well, and incubated for 1 hr at 37°C. Subsequently, each well was washed 6 times with 0.05% T20-PBS. Standard apoC-III and a sample diluted with 0.3% G-PBS were added to each well. After incubation for 2 hr at 37°C, the content of each well was aspirated and the plate was washed 6 times with 0.05% T20-PBS. To detect the antibody-bound apoC-III, a rabbit anti-bovine-apoC-III antibody biotinylated with a commercial kit (Sigma Chemical Co., U.S.A.) was diluted with 0.3% G-PBS, and dispensed into each well. Thereafter, the microplate was incubated for 2 hr at 37°C and washed 6 times with 0.05% T20-PBS. Avidin-alkaline phosphatase conjugate (Sigma Chemical Co., U.S.A.) was diluted with 0.3% G-PBS solution was added to each well. The microplate was incubated for 1 hr at room temperature, and was washed 6 times with 0.05% T20-PBS. Alkaline phosphatase substrate solution (Bio-RAD Laboratories, U.S.A.) was pipetted into each well and, after that, incubated for 1 hr at room temperature in the dark. Finally, the reaction was stopped by adding 0.4 M NaOH solution, and the developed color was read with an immunoreader (Immuno Mini NJ-2300, Inter Med, Tokyo, Japan) at an absorbance of 405 nm.

Other methods: The liver TG content was determined by the method of Snyder and Stephens [16]. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis procedure are described in the literature [22]. Serum activities of glutamic-oxaloacetic transaminase (GOT) and γ-glutamyl transpeptidase (γ-GTP), and concentrations of total bilirubin (TB), total cholesterol (TC), free cholesterol (FC), TG, phospholipids (PL) and NEFA were measured with commercial kits (Wako Pure Chemical, Osaka, Japan). The serum cholesterol ester (EC) concentration was calculated by subtracting the concentration of FC from that of TC. The serum concentration of apolipoprotein B-100 (apoB-100) was determined by ELISA [20].

Statistical analysis of data: Each assay was done in triplicate. Data were expressed as the mean (control) and the
RESULTS

The effects of ethionine administration on liver TG content, serum apoC-III and biochemical variable concentrations were examined. As shown in Fig. 1, liver TG content was remarkably increased after the first administration at days 14 (85.3 ± 37.1 mg/g) and 21 (86.4 ± 38.8 mg/g) compared with day 0 (12.8 ± 0.5 mg/g), but no change was observed in controls. On the other hand, the serum apoC-III concentration was decreased drastically and fell to the lowest value at day 10 (76.2 ± 5.3 µg/ml), 32% of the value at day 0 (235.8 ± 20.3 µg/ml). After the final administration, the apoC-III concentration did not recover immediately to the value before administration. Through almost all of the experimental period, the serum apoC-III concentration was significantly decreased (Fig. 2). SDS-PAGE and western blotting analysis were conducted with several serum samples from the cows with typical fatty liver. The reduction of the apoC-III concentration in the affected cow was also detected by this method. The density of each band well supported the reliability of sandwich ELISA (Fig. 3). ApoC-III concentrations in controls during the experiment were comparable with a previous report [21].

Linearity in the standard curve of the established sandwich ELISA was obtained from 36.9 ng/ml to 4.6 µg/ml using purified apoC-III. The detection limit (1.85 ng/well) was almost one hundred times as sensitive as the previous method [21]. The intra-assay coefficient of variance was in the range from 5.9 to 6.6%. Recovery rate values by the addition of standard apoC-III ranged from 101.9 to 103.2%. In a previous report, when serum was used as the antigen, the addition of 2-mercaptoethanol (2-ME) was required to detect apoC-III, which was present in lipoprotein particles [21]. Nevertheless, this sandwich ELISA system could obtain a reliable calibration curve without the addition of 2-

ME.

After administration, the serum activity of GOT and concentrations of NEFA were clearly increased, whereas the activity of γ-GTP was only slightly increased (Table 1). An increase in the serum TB concentration was observed, although the differences did not reach statistical significance. Concentrations of cholesterols, PL and TG were somewhat decreased. After that, the activity of GOT and concentration of TB recovered smoothly, but other biochemical variables were recovered only moderately. Although the serum apoB-100 concentration decreased

Fig. 2. Changes in serum apolipoprotein C-III (apoC-III) concentrations in control cows and cows with ethionine administration. Arrows indicate the administration of ethionine (treated, n=5) or saline (control, n=2) at days 0, 7 and 14. Serum apoC-III concentrations were determined by developed sandwich ELISA (see methods for key). * p<0.05 and ** p<0.01, compared with value at day 0. ○, control (mean); ●, treated (mean ± SEM).

Days after first administration

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Fig. 3. Western blotting analysis of serum apolipoprotein C-III (apoC-III) in a representative cow administered ethionine. A 6-µl aliquot of serum diluted 20-fold with PBS was loaded per well and then electrophoresed with the Tricine-buffer system [22]. A blotting membrane was reacted with 4,000-fold-diluted rabbit anti-bovine-apoC-III antibody and thereafter with 25,000-fold-diluted goat anti-rabbit IgG antibody conjugated with horseradish peroxidase. Purified apoC-III (3.6 µg) was run in the left lane as the standard (Std.).
Table 1. Changes in serum biochemical variables in control cows and cows with ethionine administration

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<tr>
<th>Variables</th>
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Fig. 4. Changes in serum apolipoprotein B-100 (apoB-100) concentrations in control cows and cows with ethionine administration. Arrows indicate the administration of ethionine (treated, n=5) or saline (control, n=2) at days 0, 7 and 14. Serum apoB-100 concentrations were determined by ELISA as previously reported [20]. ∗ p<0.05 compared with value at day 0. ○ control (mean); ● treated (mean ± SEM).

noticably every time the agent was administered, the concentration tended to recover smoothly (Fig. 4). No significant changes in the serum biochemical variables of controls were observed. Correlation coefficient values in the treated cows between apoC-III and other serum biochemical variables were: γ-GTP (r=-0.407, p<0.05); TB (r=-0.464, p<0.05); EC (r=0.449, p<0.05); NEFA (r=-0.526, p<0.05) and apoB-100 (r=0.601, p<0.05). On the other hand, no significant correlation was found between apoC-III and TG or PL as in a previous report [22].

DISCUSSION

In this experimental model, fatty liver was undoubtedly induced after treatment. In a previous report, fatty liver was defined as TG content exceeded 30 mg TG/g of liver (wet weight) [17]. The changes in serum biochemical variables observed in present study well reflected the process of liver functional disorder in the development of fatty liver, and these values resembled the fatty liver naturally occurring in the field [12, 13]. Repeated administrations of ethionine are known to lead to highly increased hepatic TG content [17]. Methionine, one of the essential amino acid, binds to the adenosine moiety of adenosine triphosphate (ATP) and forms S-adenosylmethionine. After that, the product is utilized for various metabolisms. Ethionine, an ethyl analog of methionine, is useless for metabolism, competes with methionine to bind to adenosine, and forms S-adenosylmethionine. The competition between methionine and ethionine results in a relative reduction of the S-adenosylmethionine concentration. Reduction of the ATP concentration as well as the reduction of methylation capacity attributed to lowering of the S-adenosylmethionine concentrations perturbs several metabolic processes, including protein synthesis in liver.

The sandwich ELISA developed for apoC-III was sensitive, and the values obtained were reliable, as indicated by the coefficient of variance. By means of the assay system, serum apoC-III concentrations were evaluated in experiment cows. By repeated administrations, the concentration of apoC-III was noticeably reduced, and was correlated negatively with serum NEFA, γ-GTP and TB and positively with EC. The negative correlations suggest that apoC-III is involved in the occurrence of fatty liver. On the other hand, the positive relationship between apoC-III and EC can be explained, at least in part, by the fact that apoC-III promotes...
the activity of LCAT, the enzyme responsible for esterification of cholesterol in plasma [6, 15]. The nonsignificant correlations between apoC-III and serum TG and PL may be due to different metabolic pathways, as previously reported in the article on apoB-100 [20].

ApoB-100, a major structural apoprotein of very-low density lipoprotein (VLDL) synthesized in liver [8], is also affected by liver functional disorders [8]. The reduction of apoB-100 production results in a disorder of lipoprotein secretion from the liver. Thereby TG is increasingly accumulated and hepatic lipidosis develops. In the present study, apoB-100 reduction was observed as in a previous report [16], but its pattern was noticeably different from that of apoC-III. Briefly, although the concentration of apoB-100 recovered smoothly after every administration, that of apoC-III remained low. This reduction in apoC-III seems to indicate the presence of other regulatory pathways. In other words, it is considered that not only is the synthesis of apoproteins inhibited by ethionine administration but also other regulatory factors are involved. It is less probable that the reduced apolipoprotein concentration was attributable to liver damage caused by biopsy because no change in serum biochemical variables in controls was observed. The synthesis of Apo-C-III in the liver is regulated by cytokines [3], retinoid X receptor (RXR) [18], peroxisome proliferator activated receptor (PPAR) [4], and hepatic nuclear factor-4 (HNF-4) [14]. Of those regulators, PPAR is considered to be one of the major regulatory factors of intracellular apoC-III synthesis. PPAR reduces apoC-III gene expression and enhances lipoprotein lipase gene expression [2]. On the other hand, it was reported that PPAR is activated by fatty acids, as well as fibrates [11]. Therefore, the significant negative correlation between apoC-III and NEFA appears to reflect the activation of PPAR by fatty acid. The reduction in apoC-III would also be directly involved in the depression of the serum TG level and the induction of remnant lipoprotein to the liver, and these changes might facilitate liver lipidosis in such conditions.

In this study, the change in the apoC-III concentration and the association of apoC-III with serum biochemical variables were shown in the development of fatty liver in dairy cattle. These suggestive findings obtained from this study are considered to be essential to elucidate the apoC-III-related lipid metabolic regulation systems in future research.

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