REVIEW *Biochemistry*

Relevance of Apolipoproteins in the Development of Fatty Liver and Fatty Liver-Related Peripartum Diseases in Dairy Cows

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ABSTRACT. Most metabolic diseases in dairy cows occur during the peripartum period and are suggested to be derived from fatty liver initially developed during the nonlactating stage. Fatty liver is induced by hepatic uptake of nonesterified fatty acids that are released in excess by adipose tissues attributable to negative energy balance. The fatty accumulation leads to impairment of lipoprotein metabolism in the liver, and the impairment in turn influences other metabolic pathways in extrahepatic tissues such as the steroid hormone production by the corpus luteum. Detailed understanding of the impaired lipoprotein metabolism is crucial for elucidation of the mechanistic bases of the development of fatty liver and fatty liver-related peripartum diseases. This review summarizes results on evaluation of lipoprotein lipid and protein concentrations and enzyme activity in cows with fatty liver and those with ketosis, left displacement of the abomasum, milk fever, downer syndrome and retained placenta. Obtained data strongly suggest that decreases in serum concentrations of apolipoprotein B-100, apolipoprotein A-I and apolipoprotein C-III, a reduction in activity of lecithin:cholesterol acyltransferase and induction of haptoglobin and serum amyloid A are intimately related to the development of fatty liver and fatty liver-related diseases. Moreover, determination of the apolipoprotein concentrations and enzyme activity during the peripartum period is useful for early diagnoses of these diseases.

KEY WORDS: apolipoprotein, bovine, estrogen receptor, fatty liver, protein kinase C.

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PREFACE AND SCOPE OF THIS REVIEW

Control of metabolic diseases, including ketosis, left displacement of the abomasum (LDA), milk fever and downer cow syndrome are crucially important for modern dairy husbandry, because their occurrence is intimately related to extensive feeding and management of dairy cows and also because high-yielding cows are particularly susceptible to such diseases. Affected cows, even after recovery from those metabolic diseases, have high incidences of reproductive and infectious diseases. Most metabolic diseases occur during the peripartum period (2 to 4 weeks from parturition) and are suggested to be caused by fatty liver (hepatic lipidosis) initially developed during the nonlactating stage. Fatty liver is a condition in which triglycerides (TG) accumulate in the liver. As a logical consequence, the TG accumulation results in impaired metabolism of lipoproteins, most of which are produced by the liver. Lipoproteins have a role in the transport of lipids between the liver and extrahepatic tissues. Impairment of lipoprotein metabolism is linked to disturbances of other metabolic pathways in extrahepatic tissues, for example, the steroid hormone production in the corpus luteum. Studies on impaired lipoprotein metabolism are therefore prerequisite to elucidate the mechanistic bases of the development of fatty liver and fatty liver-related diseases. Lipoproteins consist of lipids and apoproteins. Lipoprotein lipid concentrations are quickly altered by conditions such as time from feeding, whereas apoprotein concentrations are relatively stable, thereby providing apoprotein concentrations and enzyme activity as diagnostic markers for fatty liver and related diseases.

The purpose of this review is to summarize studies on lipoproteins in cows with fatty liver and those with related diseases. This review begins with clinico-biochemical overviews of fatty liver, ketosis, LDA, milk fever, downer cow syndrome, retained placenta and mastitis, in particular focus on unique characteristics of cattle lipid metabolism. The reason for the inclusion of retained placenta and mastitis is that their development is suggested to be associated with fatty liver. After a brief summary of the lipoprotein metabolism, we present results on alterations in apoprotein concentrations and enzyme activities in cows with fatty liver and those with related diseases. Apolipoproteins (apo) include apoB-100, apoA-I, apoC-III, lecithin:cholesterol acyltransferase (LCAT), haptoglobin (Hp) and serum amyloid A (apoSAA). Hp and apoSAA, usually categorized as acute-phase proteins, are intimately related to the lipoprotein metabolism. We also propose the usefulness of these apoproteins as diagnostic markers. Hepatic synthesis of these apoproteins is regulated by a wide variety of intracellular signal transduction systems. Possible involvement of steroid hormone receptors, nuclear receptors and protein phosphorylation on the development of fatty liver and related diseases is discussed.

FATTY LIVER

The lactation cycle of cows is mainly divided into four stages; early lactating, midlactating, late lactating and nonlactating. During 60 to 90 days after parturition in early lactation, cows are inseminated for the next lactation. The milk yield is highest during midlactation, and cows during this stage are fed high-energy diets. The feeding of such diets tends to continue even during late lactation, particularly in

high-yielding cows, and leads to shortening of and overfeeding during the nonlactating stage and, as a consequence, cows become obese before parturition. Near parturition, feed intake is reduced. On the other hand, after parturition, the demand for energy is progressively increased by the initiation of lactation. The negative energy balance attributable to the reduced feed intake and the initiation of lactation is compensated for by the mobilization of nonesterified fatty acids (NEFA) from adipose tissues. NEFA is transported by binding with serum albumin and is taken up mainly by the liver [13].

NEFA incorporated into the liver is converted to TG and is secreted as very low-density lipoprotein (VLDL) or, alternatively, is oxidized in mitochondria and peroxisomes. In cattle, unlike in rats and humans, the major site for fatty acid synthesis is adipose tissues, not the liver [13]. The ability to secrete hepatic TG as VLDL is therefore extremely low compared with nonruminant animals [147]. This unique property explains, at least in part, why fatty liver is the critical condition for dairy cows. Hepatic activity for NEFA oxidation appears to be similar between ruminants and nonruminants [85]. When the amount of incorporated NEFA exceeds the amount secreted as TG by and oxidized in the liver, TG accumulates in the liver and fatty liver develops [39, 50, 121, 122, 151]. During the peripartum period, plasma concentrations of steroid hormones are considerably altered [167] to adapt to the transition from the pregnant, nonlactating state to the nonpregnant, lactating state [41]. The hormonal alteration, particularly of estradiol (E_2) and glucocorticoids, is thought to be an additional factor for fatty liver development [121].

Hepatic TG contents in healthy cows during the peripartum period are less than 30 mg/g of liver (wet weight) and fatty liver is defined as the liver having more than 30 mg/g [73]. Fatty liver is experimentally induced by nonfeeding [25, 152], nonfeeding with administration of E_2 [46, 74] and administration of the methionine analog ethionine [173]. The nonfeeding increases the NEFA concentration. Dexamethasone administration similarly elevates the NEFA concentration [200]. The administration of E_2 to nonfed cattle mimics the condition near parturition [90]. Ethionine competes with methionine and reduces the adenosine triphosphate (ATP) concentration by trapping the adenosine moiety of ATP as *S*-adenosylethionine and thereby inhibits ATP-dependent hepatic synthesis of proteins and phospholipids (PL) [36].

In cattle, concentrations of cholesterol and PL are comparable to those in humans, whereas the TG concentration is extremely low (approximately one-tenth of humans). In addition to the limited VLDL secretion [147], the low TG concentration is attributed to the ruminal contribution to lipid metabolism. Lipid contents in the cattle diet are low (less than 5%) and the major energy source is volatile fatty acids produced in the rumen rather than saturated long-chain fatty acids absorbed by the small intestine [144]. Serum concentrations of TG, cholesterol, particularly cholesteryl esters (CE), and PL are decreased in experimental [173] and natural cases [51, 129] of fatty liver. In cows with fatty liver, fatty acid compositions in liver and plasma lipids are altered; the concentration of oleic acid (C18:1n-9) is increased whereas that of linoleic acid (C18:2n-6, a precursor for prostaglandins) is decreased [25, 157].

FATTY LIVER-RELATED DISEASES

Ketosis: Besides the conversion to TG, NEFA in the liver is β-oxidized in mitochondria and peroxisomes. Under normal conditions, the peroxisomal β-oxidation is a minor pathway for fatty acid oxidation. However, during nonfeeding, this pathway is enhanced [143]. Acetyl-coenzyme A (acetyl-CoA), the product of the β-oxidation, is further oxidized in the tricarboxylic acid (TCA) cycle by binding with oxaloacetic acid, an intermediate of the TCA cycle and also an obligatory link between the cycle and the gluconeogenesis pathway. In cows during early lactation, the demand for gluconeogenesis is prominently increased for the synthesis of milk lactose. Oxaloacetic acid is exhausted for gluconeogenesis (which occurs in cytosol) and is depleted from mitochondria. As a result, acetyl-CoA cannot enter the TCA cycle and, instead, is directed toward the pathway for ketogenesis [86]. The excess formation of ketone bodies such as β-hydroxybutyric acid in turn causes ketosis. The transport of NEFA into mitochondria is controlled by carnitine palmitoyltransferase. The regulation of the enzyme activity by malonyl-CoA is also proposed to be relevant in the development of ketosis [22].

Ketosis is experimentally induced by nonfeeding [25] and by administration of 1,3-butanediol, a ketone precursor that is absorbed by the rumen [180]. In both cases, fatty liver precedes ketosis. Fatty liver is found in almost all natural cases of ketosis [142, 163]. As in fatty liver, overfeeding is epidemiologically suspected as a major causal factor for ketosis [106]. In addition to the increase in the NEFA concentration [87], decreased concentrations in CE and PL are observed in cows with ketosis [125].

Left displacement of the abomasum: The association of fatty liver with LDA has been reported [57]. The association of ketosis is also frequently observed [142, 145]. An increase in the NEFA concentration [87] and decreases in CE and PL concentrations [125] are found in cows with LDA. Duodenal instillation of fat and protein digestion products induces abomasal atony, a prerequisite factor for the development of LDA [166]. Feeding of rations high in fat and protein and low in bulk during the nonlactating stage is believed to be the major causal factor [145]. The effect of volatile fatty acids produced by such feeding on the induction of abomasal atony is controversial [17, 20]. Hypocalcemia appears to be another risk factor for LDA, suggesting a relation to milk fever [108]. Parathyroid hormone (PTH), the calcium (Ca)-regulating hormone, reduces the motility of reticulo-ruminal smooth muscle [27].

Milk fever: Milk fever is characterized by recumbency attributable to hypocalcemia [4]. Most cases of milk fever occur earlier (before parturition or within a few days after

calving) than ketosis and LDA. An elevated NEFA concentration [97] and fatty liver [56] are associated with milk fever. Concentrations of CE and PL are greatly decreased [192]. In rat hepatocytes, the secretion of CE as lipoproteins is regulated by Ca [109]. The TG concentration is not decreased, but rather increased in cows with milk fever [192]. This increase cannot be explained simply by the interruption of milking by recumbency, because, in addition to TG [40, 177], CE is taken up by the mammary gland [118]. Cows with milk fever have higher E_2 concentrations than healthy cows [4, 161]. Administration of E_2 enhances the VLDL secretion [26, 184]. In rats, Ca deficiency modifies the polyunsaturated fatty acid metabolism by reducing liver desaturase activity [107].

Downer cow syndrome: This syndrome is defined by the following criteria; cows are recumbent, but have Ca concentrations similar to healthy cows and do not respond to Ca treatment [4]. Fatty liver is associated with downer cows [4]. In a study using slaughtered cows, nearly 70% of downer cows were found to have fatty liver [140], suggesting that fatty liver is one of the causal factors. To adapt to accelerated gluconeogenesis during early lactation, amino acids such as aspartic acid are mobilized in excess from skeletal muscle, the major pool of amino acids [86]. The abundant amino acid loss from skeletal muscle may induce recumbency.

Retained placenta: A placenta not expelled within 24 hr (or 12 hr) after delivery is the definition of this disease [171]. Although retained placenta is experimentally induced by the removal of the corpus luteum [111], decreases in serum concentrations of E_2 and progesterone (P_4) near parturition are not directly related to the development [1]. The association of fatty liver [122] and hypocalcemia [32] has been reported. A possible relation to overfeeding has also been suggested [31]. An increase in NEFA and decreases in CE and PL concentrations are observed in cows with retained placenta [139].

In reproductive diseases other than retained placenta, fatty liver is associated with infertility [39, 153]. During early lactation, cows with fatty liver have lower serum P_4 concentrations than healthy cows [185]. Because CE is used for the synthesis of P_4 in the corpus luteum, an insufficient CE supply to this steroidogenic tissue may cause infertility.

Mastitis: Reduced immune competence is observed in cows with fatty liver [201], ketosis [158], LDA [48], milk fever [154] and retained placenta [47]. In experimental mastitis, fatty liver increases the severity [54]. Epidemiological studies indicate the close association of metabolic and reproductive diseases with mastitis developed during early lactation. For example, odds ratios for mastitis are reported to be 5 to 8 for milk fever [31, 32] and 4 for retained placenta [160]. The odds ratio means that cows with milk fever are 5 to 8 times more likely to develop mastitis than cows without milk fever. Near parturition, the glucocorticoid concentration is markedly increased [167]. Glucocorticoids reduce prostaglandin synthesis by suppressing phospholipase A_2 activity [202]. In cows, glucocorticoids inhibit expression of proinflammatory cytokines in macrophages [60, 72] and down-regulate glucocorticoid receptor concentrations in leukocytes [146]. It appears that the elevation of the level of this steroid hormone during the peripartum period has bifunctional effects on fatty liver development and immunosuppression. Hypocalcemia has also been suggested to act as an immunosuppressor by decreasing the production of polyunsaturated fatty acids [107], the precursors for prostaglandins.

A BRIEF SKETCH OF LIPOPROTEIN METABOLISM

Lipoproteins are mainly classified into five subfractions; chylomicrons (CM, d<0.95), VLDL (d<1.006), low-density lipoprotein (LDL, d<1.063), high-density lipoprotein (HDL, d<1.21) and very high-density lipoprotein (VHDL, d<1.25) [99]. Apoproteins in HDL, LDL and VLDL fractions are alphabetically named A, B and C apolipoproteins, respectively [2].

CM is produced by the small intestine and is the carrier for dietary lipids. The major apoprotein in CM is apoB-48. CM TG is hydrolyzed by lipoprotein lipase, which is distributed in endothelial surfaces, and fatty acids liberated are used as an energy source by various tissues. In cows, CM is utilized by the mammary gland to produce milk fats [177]. The CM remnants, formed after hydrolysis, are taken up by the liver.

VLDL is of liver origin and is responsible for the transport of TG and cholesterol to extrahepatic tissues. ApoB-100 is the major apoprotein in VLDL. VLDL TG is hydrolyzed by lipoprotein lipase, as in the CM TG. After the hydrolysis, VLDL is converted to VLDL remnants (intermediate-density lipoprotein). The remnants are further metabolized to LDL by receiving CE from HDL. LDL is finally taken up mainly by the liver and steroidogenic tissues such as the corpus luteum, via LDL receptors located on the cell surface [23]. LDL apoB-100 acts as the ligand for the LDL receptor. LDL CE is hydrolyzed by lysosomal acid lipase to free cholesterol (FC) and fatty acids, and FC is used as a source for synthesis of bile acids in the liver and P_4 in the corpus luteum.

HDL is a mixture of the nascent HDL particles secreted by the liver and the small intestine and also of the surface remnants produced after CM hydrolysis. HDL is rich in cholesterol and PL and its major apoproteins are apoA-I and apoC-III. HDL takes up FC from extrahepatic tissues. Then HDL FC is converted to CE by LCAT that is activated by apoA-I [64]. HDL CE is thereafter transferred to LDL by cholesteryl ester transfer protein (CETP). LDL is taken up by the liver or by steroidogenic tissues as described above. The cholesterol transport from extrahepatic tissues such as the heart to the liver is called reverse cholesterol transport. VHDL is rich in PL and serum albumin but its physiological role has not yet been clearly elucidated.

APOLIPOPROTEINS

Apolipoprotein B-100: ApoB consists of apoB-100 (512 kDa and 4,536 amino acid residues in humans) and apoB-48 (2,152 residues) [28]. ApoB-48 is the N-terminal 48% of the full-length apoB-100 and is produced by posttranscriptional editing of apoB mRNA [10]. In the rat, apoB-48 is produced by both the liver and small intestine. Because the cow liver, as in humans, lacks editing activity [43], apoB-48 is not synthesized in the liver. The serum concentration of cow apoB-48 has been determined [178].

ApoB-100 is the TG-binding protein in VLDL. The synthesis of apoB-100 is regulated almost exclusively at the posttranslational level. The amount of newly synthesized apoB-100 exceeds its secretion as VLDL. The hepatic content of apoB-100 is controlled by intracellular proteolytic degradation [197]. In addition to apoB-100 and TG, microsomal triglyceride transfer protein [94], FC, CE, phosphatidylcholine (PC) [198] are required for VLDL assembly. The requirement of PC appears to be related to experimental fatty liver induced in rats by choline deficiency [95]. In birds, the secretion of apoB-100 is enhanced by the synthetic estrogen diethylstilbestrol [26]. Although the estrogen effect is not evident in mammals, the rate of VLDL secretion is higher in female than in male rats [184].

Cow apoB-100 was isolated electrophoretically and the serum apoB-100 concentration was evaluated by single radial immunodiffusion [74, 102] and by enzyme-linked immunosorbent assay (ELISA) [194]. Reported cattle serum apoB-100 concentrations are in the range of 50 to 200 μg/m*l* and are considerably lower than those in humans (0.8 to 1 mg/m*l* [99]). The lower apoB-100 concentration is consistent with the low TG concentration in cows and appears to reflect the limited VLDL secretion [147]. In the lactation cycle, the apoB-100 concentration is low during early lactation, relative to the other stages [103, 194]. However, the hepatic concentration of apoB-100 mRNA does not significantly decrease during early lactation [45], suggesting that the apoB-100 concentration during early lactation is regulated at posttranslational levels such as the intracellular proteolytic degradation.

Reduction of VLDL and LDL lipid concentrations in cows with fatty liver was first reported by Herdt *et al.* [51] and then by Rayssiguier *et al.* [148]. The apoB-100 concentration was decreased in cows with fatty liver induced by ethionine [173] and those with natural fatty liver [104]. We found that the apoB-100 concentration in steers was increased by administration of E_2 and was decreased by subsequent nonfeeding, together with cessation of E_2 administration [74]. The rapid decline of the apoB-100 concentration, which mimics the change during the peripartum period, resulted in the development of fatty liver. In this study, the hepatic estrogen receptor concentration was evaluated to assess the E_2 effect on the apoB-100 concentration. The receptor concentration was concomitantly increased by the administration of E_2 and progressively decreased by nonfeeding and the cessation of E_2 administration. The decrease in the estrogen receptor concentration was reproduced in ethionine-induced fatty liver in the rat [79]. In cows with E_2-induced fatty liver, the serum TG concentration was decreased in association with the apoB-100 concentration. The rapid alteration of the E_2 concentration may enhance the intracellular degradation of apoB-100 and in turn reduce VLDL assembly or secretion.

The apoB-100 concentration is decreased in cows with ketosis [142], LDA [142], retained placenta [139], milk fever [140] and downer syndrome [140]. The decreased apoB-100 concentrations are similar among all diseased cows (40 to 60% of healthy controls during early lactation) and, moreover, are not largely different from that in cows with fatty liver. Similar decreased rates suggest that decreases of apoB-100 concentrations in ketosis, LDA, retained placenta, milk fever and downer cow syndrome are primarily attributable to fatty liver, thereby supporting the hypothesis that these diseases are derived from fatty liver [39, 50, 121, 122, 151].

Apolipoprotein A-I: ApoA-I in humans is a single polypeptide of 243 amino acids and its calculated molecular mass is 28.1 kDa [37]. ApoA-I is produced by both the liver and the small intestine and the contributions to apoA-I synthesis by the two organs are nearly equal in the rat [187]. In plasma, apoA-I is distributed in HDL and CM. HDL apoA-I has an essential role in cholesterol transport by activating LCAT activity [64]. Linkage of apoA-I and apoC-III has been shown in human chromosome 11 [67]. ApoA-I has affinities to Hp [88] and annexins [24].

In cattle, apoA-I is a major apoprotein in the HDL fraction [30, 62]. The cloning [137] and amino acid sequence (241 residues) [168] of cattle apoA-I have been reported. Serum concentrations of apoA-I, determined by single radial immunodiffusion [110] and ELISA [138], are in the range of 0.5 to 1.5 mg/m*l* and are comparable to those of humans (1 to 1.5 mg/m*l* [99]). The cow apoA-I concentration is not largely altered during the lactation cycle. The secretion of apoA-I by calf liver parenchymal cells has been reported [117]. The concentration of apoA-I (but not apoB-100) is decreased in cows with diarrhea [141], indirectly suggesting that cow apoA-I is also synthesized by the small intestine. Other than the liver and the small intestine, cattle apoA-I is produced by the corpus luteum [131]. In a calf with hyperlipidemia [193], the apoA-I concentration was increased compared with healthy controls, and apoA-I was detected together with Hp in CM and HDL fractions, suggesting the affinity with Hp. The detection of annexins in bronchoalveolar lavage fluids from calves with experimental pneumonia [75] may indicate the affinity of apoA-I to annexins, because HDL lipids are used for the synthesis of pulmonary surfactants [182].

The apoA-I concentration is decreased in cows with ethionine-induced [173] and naturally acquired fatty liver [104], and those with ketosis [142], LDA [142], retained placenta [139], milk fever [140] and downer syndrome [140]. The decreased apoA-I concentrations (60 to 80% of healthy controls) are similar in all these diseases, including

fatty liver. The indistinct decreases, compared with those in the apoB-100 concentration (40 to 60% of controls), may support the intestinal synthesis of cattle apoA-I. The intestinal synthesis appears not to be considerably affected in diseased cows, because the apoA-I decrease is not distinct in cows with LDA.

Apolipoprotein C-III: Human apoC-III consists of an 8.75 kDa polypeptide chain of 79 amino acid residues with a single carbohydrate side chain containing 1 mole each of galactosamine and galactose and 0 to 2 moles of sialic acid [21]. ApoC-III is synthesized in the liver and in minor quantities by the small intestine [133]. Hepatic synthesis of apoC-III is regulated by cytokines [44] and, also by retinoid X receptor (RXR) [183], peroxisome proliferator-activated receptor (PPAR) [52] and hepatic nuclear factor-4 (HNF-4) [149]. ApoC-III is distributed in human plasma in the CM, VLDL and HDL fractions [99, 133]. ApoC-III is associated with HDL in the fasting state, but is transferred to CM and VLDL during absorption of dietary fat by the small intestine [49]. ApoC-III is involved in the regulation of the TG metabolism by suppressing the hepatic uptake of the CM remnants [164]. The total plasma concentration of apoC-III is increased in patients with hypertriglyceridemia [68]. In mice, overexpression of the apoC-III gene results in hypertriglyceridemia [61], whereas disruption of the apoC-III gene induces hypotriglyceridemia [98].

The partial primary structure of cattle apoC-III has been reported [15]. We purified two species (8.2 kDa and 7.3 kDa) of cow apoC-III with an identical N-terminal amino acid sequence [190]. The 7.3 kDa apoC-III was the major species. The serum concentration of cow apoC-III was determined by ELISA, and in the lactation cycle, the apoC-III concentration showed the lowest value (less than 100 μg/m*l*) during the nonlactating stage, gradually increased during early lactation, and reached the maximum concentration (more than 100 μg/m*l*) during midlactation [191]. The value during midlactation is comparable to the reported concentration in humans [68, 99, 133]. ApoC-III concentrations in 2- to 3-month-old male Holstein calves [188] and Japanese black calves [193] were in the range of 20 to 40 μg/m*l* and were lower than those in Holstein dairy cows. The apoC-III concentration was decreased in Holstein calves with pneumonia [188], whereas greatly increased in a Japanese black calf with hyperlipidemia [193].

In contrast to its human counterpart, cattle apoC-III has a unique property with respect to the distribution in lipoprotein fractions; the apoC-III is detected mainly in HDL, but only faintly in the CM and VLDL fractions, independent of time from feeding [71, 190]. Except for the calf with hyperlipidemia (higher concentration in the CM than HDL fraction) [193], the predominant distribution in HDL does not change in pathologic states such as calf pneumonia [188] and cow ketosis and milk fever [192]. The low apoC-III concentration in CM appears to be due to the indistinct alimentary lipemia [144]. The faint detection of apoC-III in VLDL seems to result from limited VLDL secretion [147].

The apoC-III concentration is decreased in cows with fatty liver, ketosis, LDA, milk fever and retained placenta compared with healthy cows during early lactation [192]. Unlike apoB-100 and apoA-I, the decreased apoC-III concentration is distinct in milk fever among all the diseases examined, suggesting the particular relevance of apoC-III in the development of milk fever.

The plasma Ca concentration is mainly controlled by vitamin D and PTH [4, 59]. A decrease in the Ca concentration during the peripartum period stimulates PTH secretion. PTH in turn increases the formation of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], and this active form of vitamin D accelerates intestinal Ca absorption. In cows with milk fever, neither 1,25-$(OH)_2D_3$ nor the PTH concentration is decreased [59]. The 1,25-$(OH)_2D_3$ exerts its function by binding with the nuclear vitamin D receptor (VDR). The ligand-bound VDR becomes active when it forms a heterodimer with RXR [101]. The ligand for RXR is 9-*cis*-retinoic acid. The interaction of the VDR-RXR heterodimer with vitamin D-response elements is regulated by both 1,25-$(OH)_2D_3$ and 9-*cis*-retinoic acid [172]. The RXR, independent of VDR, forms a homodimer or a heterodimer with PPAR [101]. The PPAR is activated by peroxisome proliferators including fibrates (hypolipidemic drugs) and by fatty acids such as docosahexaenoic acid (C22:6n-3) [82]. Retinoids [183] increase hepatic expression of apoC-III whereas fibrates and fatty acids [159] decrease it. In addition, HNF-4, another nuclear receptor, controls the hepatic apoC-III expression [52, 149]. HNF-4 also interacts with PPAR [52]. The serum E_2 concentration is increased in cows with milk fever [4, 161]. E_2 modulates the signal transduction system mediated by RXR and PPAR [81]. Considering all these results together, it is possible to assume that milk fever is caused by impaired linkage between the Ca-regulating system and the apoC-III metabolism. The RXR appears to have a key role in the linkage. More detailed studies on apoC-III, together with those on the NEFA composition [25, 157], vitamin A [42], vitamin D [59], PTH [59] and E_2 [161] may shed light on the mechanistic basis for the development of milk fever.

Elevation of the apoC-III concentration increases the TG concentration by suppressing the hepatic uptake of CM remnants [164], as observed in hypertriglyceridemia [61, 68, 193], whereas its decline results in a decreased TG concentration [98]. The TG concentration is not decreased but rather increased in cows with milk fever, in spite of a distinct reduction in the apoC-III concentration. This discrepancy may be explained by the assumption that the acceleration of VLDL secretion by E_2 [184] is superior to an increase in the uptake of CM remnants caused by the decreased apoC-III concentration.

ApoC-III concentrations of 17 cows were monitored during the peripartum period [77]. Of the 17 cows, 14 were apparently healthy during the period. ApoC-III concentrations in healthy cows around parturition, compared with those at 21 days before parturition, were increased in 5 cows but decreased in 9 cows. The increase in the 5 cows appeared to reflect the transition of the apoC-III concentra-

tion from the nonlactating to midlactating stages [191]. Three cows had milk fever at around parturition and their decreased apoC-III concentrations were much more distinct than those in the 9 healthy cows. In the 9 healthy cows, the apoB-100 concentrations were also decreased compared with the 5 cows with increased apoC-III concentration. The 9 cows with decreased apoC-III and apoB-100 concentrations, although they did not show any distinct signs of diseases during the observed period, may be categorized as a highly susceptible group for fatty liver-related diseases.

Hypocalcemia is postulated as a risk factor for LDA [108]. The decrease in the apoC-III concentration in cows with LDA was not as distinct as in cows with milk fever. The indistinct decrease in LDA is explained by the assumption that the involvement of apoC-III is restricted to an early stage (before the appearance of clinical signs) of LDA development, because milk fever usually precedes LDA.

Lecithin:cholesterol acyltransferase: LCAT is the enzyme catalyzing the transfer of unsaturated fatty acids at the *sn*-2 position of lecithin (PC) to FC and producing CE and lysoPC [63, 169]. The esterification of FC by LCAT is an obligatory step in reverse cholesterol transport, and patients with LCAT deficiency show an almost complete lack of CE in plasma and a high concentration of plasma FC, and also excess accumulation of cholesterol in tissues such as the kidney [136]. Human LCAT has a single polypeptide mass of 47 kDa and 416 amino acids and is heavily glycosylated [3]. LCAT proteins, mainly of hepatic origin, have been cloned for several species, including humans [112], but cattle LCAT has not yet been. Fibrates, peroxisome proliferators, reduce the hepatic LCAT mRNA levels [170]. Lipopolysaccharides and tumor necrosis factor decrease the plasma LCAT concentration [35]. A reduction in LCAT activity with a concomitant depletion of CE, the product of the LCAT reaction, has been reported in lipopolysaccharide-administered African green monkeys [8]. The administration of ethionine reduces rat plasma LCAT activity [96]. A method for measuring LCAT activity using a liposome has been developed [100].

Cattle LCAT has been shown to have high specificity for linoleic acid at the *sn*-2 position of PC [135]. Linoleic acid is the major fatty acid in CE and PC fractions when cattle are given a diet of hay and a concentrate mixture [119]. We [176] have found that LCAT activity in cow serum is associated with the HDL fraction and is severalfold higher (near 1,000 U) than that in human serum (approximately 400 U [100]). The higher LCAT activity in cattle may be related to the much higher proportion of CE in the total plasma lipids compared with that in nonruminants [135]. LCAT activity is reduced in experimental [126] and natural cases [127] of calf pneumonia, suggesting the involvement of cytokines in its expression. LCAT activities in calves (500 to 1,000 U) are not largely different from those in cows. In diseased calves, serum CE concentrations, particularly that in the HDL fraction, are concomitantly decreased.

LCAT activity and the CE concentration are reduced in cows with ethionine-induced fatty liver [176] and naturally acquired fatty liver [128]. As expected, LCAT activity, together with the CE concentration, is similarly reduced in cows with ketosis [125], LDA [125] and milk fever [130]. The mechanism involved in the reduction of LCAT activity in cows with fatty liver and related diseases are probably multiple. The most likely explanation is the reduction in hepatic synthesis or secretion of LCAT. Decreased concentrations of apoA-I (the activator of LCAT) and FC and PL (both are the substrates for LCAT) by reduced HDL secretion are also involved. In addition, decreases in HDL apoA-I content caused by association of apoSAA with HDL (*see apoSAA*) and in linoleic acid contents of CE and PC fractions may participate in the reduction of LCAT activity.

In a time-course study during the peripartum period, it became apparent that the reduction in LCAT activity was detected prior to the occurrence of ketosis or milk fever [130]. In addition, in the same study, healthy cows during the peripartum period were found to be classified into two groups; one had unaltered LCAT activity whereas the other had reduced activity around parturition. The concentration of CE was similarly decreased in healthy cows with reduced LCAT activity, but not in those with unaltered activity. As a diagnostic marker for fatty liver-related diseases, LCAT activity is more useful than apoB-100 and apoC-III concentrations because the reduction precedes clinical signs such as ketonuria or recumbency and also because the activity is not altered during the peripartum period, at least in some healthy cows. Apparently healthy cows with reduced LCAT activity are inferred to be more susceptible to fatty liver-related diseases than those with unaltered activity [125].

LCAT activity is not reduced in cows with mastitis that occurs during early to midlactation [127], indicating that the reduction in LCAT activity is not directly related to the development of mastitis. The reduction in the LCAT activity appears to lower immune competence by sustained suppression of cholesterol transport to immune-related tissues and cells, thereby leading to the induction of mastitis during the peripartum period.

Haptoglobin: Hp is a multifunctional acute-phase glycoprotein capable of binding hemoglobin (Hb) [33]. Hp consists of a tetrameric structure composed of two α- and two β-subunits that are linked by disulfide bonds. The major site of Hp synthesis is the liver, and the hepatic synthesis is regulated by cytokines and glucocorticoids [105]. Hp synthesis is also controlled by peroxisome proliferators [7] and hepatic RXR mRNA and protein levels are suppressed during the acute-phase [12]. The plasma concentration of Hp is increased by inflammation, and there have been numerous reports on elevated Hp concentrations in various inflammatory diseases, including cattle diseases [5, 120].

Apart from inflammation, Hp is suggested to be relevant in lipid metabolism. In addition to the liver, Hp is synthesized by adipocytes [38]. The Hp locus in human chromosome 16 is located close to the loci of LCAT and CETP [150], and the human Hp subtype patterns correlate with lipoprotein lipid and protein concentrations [19]. The poly-

unsaturated fatty acids eicosapentaenoic acid (C20:5n-3) and γ-linolenic acid (C18:3n-6) directly modulate hepatic Hp synthesis [186]. Hp inhibits the synthesis of prostaglandins [65] and, conversely, the serum Hp concentration is influenced by prostaglandins [165]. Hp has an affinity to apoA-I [88]. Hp acts as an anti-oxidant for LDL [115]. Biliary Hp promotes cholesterol crystallization [195].

Unlike in humans, Hp is undetectable in serum from healthy cattle. We found 35- and 23-kDa proteins in sera of cows with fatty liver [162]. These proteins, not detected in healthy cows, were identified as Hp [199]. Cattle Hp consists of a β-chain (35 kDa) and α-chain (23 kDa). The non-inflammatory induction of Hp was first reported in cattle with transportation exhaustion [116]. The association of Hp with fatty liver was confirmed by experiments on the induction of Hp by ethionine [174], glucocorticoids [53, 200] and nonfeeding [78]. The appearance of Hp in cows around parturition also suggests the relation to fatty liver [175]. Hp was detected in sera from cows with ketosis, LDA, milk fever, downer syndrome and retained placenta (S. Oikawa, unpublished results). In diseased cows, erythrocyte and leukocyte counts and the serum Hb concentration were in normal ranges, suggesting that Hp in diseased cows is not attributable to inflammation. In sera from cows with fatty liver, Hp and Hb are detected, in addition to the lipoprotein-deficient fraction (d>1.25), in HDL and VHDL fractions [76]. The association of Hp in HDL and VHDL fractions is also found in sera from cows with mastitis [66]. In a calf with hyperlipidemia, Hp, together with apoA-I, was detected in both CM and HDL fractions [193]. The functional roles of Hp, Hb or its complex in the lipoprotein fractions have not yet been clarified.

Like infection and trauma, the fatty infiltration appears to be a harmful stimulus for liver parenchymal cells. The cells thereby seem to produce the acute-phase protein Hp. More mechanistically, the RXR- and PPAR-mediated pathway regulates the acute-phase reaction [12] and modifies hepatic Hp expression [7]. The altered concentration and composition of NEFA [25, 157] may evoke the RXR- and PPAR-mediated Hp synthesis. Hp is induced by E_2 [53] as well as by glucocorticoids. E_2 [81] and glucocorticoids [92] regulate the RXR- and PPAR-mediated pathway. The hepatic Hp production in fatty liver and fatty liver-related diseases may be regulated singly by E_2, glucocorticoids, RXR and PPAR, or by combinations of them.

Hp in cows with fatty liver and related diseases may act as a bacteriostat [34] or, although this is controversial, an inhibitor of neutrophil functions [155]. It is also possible that Hp is required for liver regeneration by scavenging TG-accumulated cells. The Hp and Hb complex induces apoptosis of hepatocellular carcinoma cells [83].

Serum amyloid A: ApoSAA is an acute-phase protein of liver origin and is also a serum precursor for tissue amyloid A protein found in tissue deposits in patients with amyloidosis [179]. Synthesis of hepatic apoSAA mRNA and protein is induced by cytokines and glucocorticoids [179]. ApoSAA is associated in plasma with HDL [14]. The association with HDL facilitates the FC efflux to injured tissues in which cholesterol is required for repair and regeneration [93]. HDL apoSAA also acts as a scavenger for cholesterol liberated in excess at inflammatory sites. The association of apoSAA with HDL results in a decrease of HDL apoA-I content, and thereby reduces LCAT activity [29]. In addition to the lipoprotein metabolism, apoSAA has immune-related functions [179].

The amino acid sequence of cattle apoSAA (112 residues) has been determined [156]. Purification [58], measurement by ELISA [18], induction by cytokines [6] and increased concentrations in inflammatory diseases [5] of cattle apoSAA have been reported. During the course of purification of cow serum apoC-III, we co-purified a 14 kDa protein and it was identified as apoSAA by N-terminal sequence analysis [189]. Besides the induction in calves with pneumonia [189], apoSAA was detected in nonfed calves [78] and cows with fatty liver, ketosis, LDA, milk fever and retained placenta (N. Katoh, unpublished results). Almost all apoSAA induced was associated with HDL. In diseased cows, apoSAA has been suggested to accelerate the CE transport to extrahepatic tissues; however, the association with HDL may decrease HDL apoA-I contents and in turn reduce LCAT activity.

Other apolipoproteins: Cattle apoE has been shown to be present in the corpus luteum [131]. ApoA-IV has been detected in the HDL fraction [71]. Amino acid sequences of apoA-II [123] and apoC-II [15] have been reported. Cloning and sequencing of apoC-I cDNA have also been done [196]. The relevance of these apolipoproteins in the pathogenesis of fatty liver and related diseases has not yet been examined. The presence of plasma CETP is not known.

PROTEIN KINASE C

Recent evidence has suggested that the signal transduction pathways mediated by the estrogen receptor [114], glucocorticoid receptor [202] and PPAR [91] are modulated by protein kinases, including protein kinase C (PKC). PKC is an enzyme activated by diacylglycerols (the precursor of TG), PL and Ca, and regulates a wide variety of biological functions by phosphorylating multiple protein and enzyme substrates at serine and threonine residues [89, 134]. Other than the three cofactors, PKC is directly activated by oleic acid [124]. PKC is distributed in nuclei as well as in other intracellular organelles [84]. PKC phosphorylates apoA-I [55] and apoSAA [132] and also phosphorylates and modulates the activity of 3-hydroxy-3-methylglutaryl CoA reductase [11], the rate-limiting enzyme of cholesterol synthesis, and of phospholipid methyltransferase [181], the enzyme catalyzing the conversion from phosphatidylethanolamine to PC. It is involved in the regulation of VLDL secretion [16], LDL receptor expression [9] and FC uptake by HDL [113]. PKC activity is inhibited by palmitoylcarnitine [80], the product of carnitine palmitoyltransferase. PKC has also been suggested to contribute to the induction of Hp [53, 200].

In a time-course experiment using ethionine-induced rat fatty liver, it was found that PKC activity is initially increased but is thereafter reduced [69]. The initial increase (found as early as 4 hr after administration) precedes the hepatic accumulation of TG (detected at 24 hr after), suggesting that the activation of PKC is an early event in fatty liver development. The initial increase in PKC activity is followed by enhanced phosphorylation of endogenous PKC substrates, including 22 kDa and 19 kDa proteins. Enhanced PKC-dependent phosphorylation is also detected prior to the accumulation of TG in the liver [69]. In ethionine-induced [70] and naturally acquired fatty liver in the cow [73], hepatic PKC activity is reduced. The reduced activity appears to correspond to the reduction observed after the initial increase of PKC activity in the rat (in ethionine-treated cows, liver samples were obtained more than 10 days after administration). The 22- and 19-kDa PKC substrates are similarly detected in livers from ethionine-administered cows [70] and dexamethasone-administered nonfed cows [200], although their phosphorylation is reduced compared with controls, as with PKC activity. These results, coupled with known properties of PKC, suggest that the activation of PKC (presumably evoked by an increased concentration of oleic acid or diacylglycerols) and the resultant enhancement in PKC-dependent protein phosphorylation occur in an early stage of fatty liver development. The activation of the PKC system may be involved in the modulation of functions of estrogen receptors, glucocorticoid receptors and PPAR. Subsequent reduction in PKC activity (caused, for example, by accumulated palmitoylcarnitine or hypocalcemia) is probably linked to the deterioration of fatty liver and, moreover, to the development of fatty liver-related diseases.

CONCLUSIONS

As depicted in Fig. 1, fatty liver is initially induced by the mobilization of NEFA. The excess infiltration of NEFA results in the accumulation of TG. Accumulated TG impairs hepatic VLDL assembly and secretion. Rapid alterations of E_2 and estrogen receptor concentrations accelerate the impairment. Concomitant with reduced VLDL secretion, the LDL apoB-100 concentration is decreased. The decrease in the apoB-100 concentration lowers the CE uptake by the liver and the corpus luteum. The insufficient CE supply further disturbs lipoprotein production in the liver and reduces the P_4 production in the corpus luteum. A decreased P_4 concentration may be linked to retained placenta and infertility.

The TG accumulation also impairs the hepatic HDL secretion. In addition to reduced hepatic secretion of LCAT, the decrease in the apoA-I concentration also reduces LCAT activity, thereby suppressing the CE transport to LDL. The association of apoSAA with HDL facilitates the reduction of LCAT activity by further decreasing apoA-I content. The association of Hp with HDL may also influence LCAT activity by binding with apoA-I. The concentration of apoC-III, which also interacts with apoA-I at the gene level, is distinctly decreased in cows with milk fever. In combination with hypocalcemia, the decreased

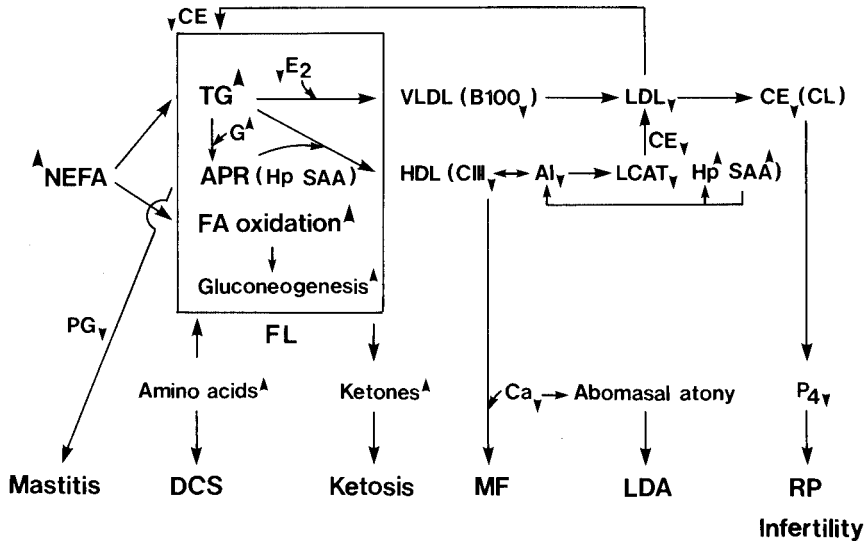

Fig. 1. Possible involvement of apolipoproteins in the development of fatty liver (FL), ketosis, LDA, milk fever (MF), downer cow syndrome (DCS), retained placenta (RP), infertility and mastitis. APR, acute-phase reaction; CL, corpus luteum; FA, fatty acid; G, glucocorticoids; and PG, prostaglandins. Other abbreviations are as in the text, except for the deletion of apo for convenience. Arrowheads show increases or decreases of metabolites and arrows indicate directions of lipoprotein metabolism, development of diseases, or the interaction between two apoproteins.

apoC-III concentration appears to be related to the development of milk fever. Hypocalcemia possibly participates in the development of LDA by inducing abomasal atony.

NEFA taken up in excess by the liver is oxidized in mitochondria and peroxisomes. Increased demand for glucose production enhances gluconeogenesis. The resultant oxaloacetic acid deficiency leads to the production of ketone bodies and ketosis. To adapt to increased gluconeogenesis, amino acids are mobilized from skeletal muscle. This mobilization may be involved in the development of downer cow syndrome. Accumulated TG or infiltrated NEFA induces an acute-phase reaction. The increased glucocorticoid concentration stimulates this reaction. The inhibition of prostaglandin synthesis, one of the functions of Hp, may be connected with the development of mastitis.

The initial increase of PKC activity and the PKC-dependent protein phosphorylation and their subsequent reductions may be related to events occurred in several stages of the development of fatty liver and related diseases.

From the practical aspect, apoB-100, apoA-I and apoC-III concentrations and LCAT activity are useful markers for early diagnoses of fatty liver and related diseases. The apoB-100 concentration reflects the difference of the amount of hepatic VLDL secretion and of CE uptake by the liver and steroidogenic tissues. The apoA-I concentration and LCAT activity are indicators of CE transport. LCAT activity is reduced prior to the occurrence of ketosis or milk fever. The apoC-III concentration mirrors the amount of hepatic HDL secretion and, although not evident in cattle, reflects the amount of hepatic uptake of CM remnants, and its evaluation is particularly relevant in diagnosing milk fever. Measurements of Hp and apoSAA concentrations are helpful to detect cows with fatty liver, as well as those with fatty liver-related inflammatory diseases, including mastitis. Monitoring of these apoprotein concentrations and enzyme activity during the peripartum period is highly valuable to detect cows susceptible to metabolic, reproductive and inflammatory diseases.

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Abbreviations: apo, apolipoprotein; apoSAA, serum amyloid A; ATP, adenosine triphosphate; Ca, calcium; CE, cholesteryl esters; CETP, cholesteryl ester transfer protein; CM, chylomicron; CoA, coenzyme A; E_2, estradiol; ELISA, enzyme-linked immunosorbent assay; FC, free cholesterol; Hb, hemoglobin; HDL, high-density lipoprotein; HNF-4, hepatic nuclear factor-4; Hp, haptoglobin; LCAT, lecithin:cholesterol acyltransferase; LDA, left displacement of the abomasum; LDL, low-density lipoprotein; NEFA, nonesterified fatty acids; $1,25\text{-}(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; P_4, progesterone; PC, phosphatidylcholine; PKC, protein kinase C; PL, phospholipids; PPAR, peroxisome proliferator-activated receptor; PTH, parathyroid hormone; RXR, retinoid X receptor; TCA, tricarboxylic acid; TG, triglycerides; VDR, vitamin D receptor; VHDL, very high-density lipoprotein; VLDL, very low-density lipoprotein.