A High Cholesterol/Cholate Diet Induced Fatty Liver in Spontaneously Hypertensive Rats

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A high cholesterol diet was found to induce fatty liver in spontaneously hypertensive rats. Although cholesterol ester and triacylglycerol accumulated in large amounts in liver, the increases of these lipids in plasma were relatively small and no increase in cholesterol and cholesterol ester was observed in aorta. In rats fed normal diet, plasma cholesterol ester mainly consisted of arachidonate species; however, oleate and linoleate esters became the most prominent species in rats fed a high-cholesterol diet. The amounts of oleate and linoleate at the 2-position of phosphatidylcholine in both plasma and liver were increased slightly, but the fatty acids of aorta lipids changed little by feeding a high cholesterol diet. These results indicate that the livers of rats fed the high cholesterol diet do not secrete cholesterol ester and triacylglycerol with altered fatty acids as rapidly as they are synthesized and that the increased levels of cholesterol oleate in liver and plasma are not directly correlated with atherogenic lesions under these conditions.
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Spontaneously hypertensive (SH) rats, a strain originally developed by Okamoto and Aoki (1) have been used widely in hypertension research (2,3). Some abnormalities in lipid metabolism have also been noted in these rats (4-9); for example, when fed atherogenic diets, SH rats develop hypercholesterolemia more easily than conventional strains (4,7). In human atherosclerotic lesions, the major lipid component has been shown to be cholesterol ester (ChoE) (10-17). ChoE present in lesions has a higher oleate and lower linoleate content than ChoE present in uninvolved regions of the artery (10-18). Similar changes in the fatty acid compositions of hepatic lipids have been observed in experimental animals (19-24). It has been proposed that ChoE hydrolase is involved in ChoE accumulation (25-27), although there is some controversy about this hypothesis (28-32). Elevations of acyl-CoA:cholesterol acyltransferase (ACAT) occur in rats and rabbits fed high cholesterol diets (33-42) while, in monkey, increases in endogenous cholesterol but not ACAT activity have been reported (43). Some differences in the levels of other enzymes have also been noted in both SH and normotensive rats: lower hepatic cholesterol synthesis in SH than in Wistar/Kyoto (WKY) rats when both were fed an atherogenic diet (6); higher biliary secretion of cholate in SH than in WKY rats fed either a normal or a high cholesterol diet (7); and higher prostanoid synthesis (44,45) and higher phospholipid turnover in aorta (46,47) of SH rats than that of WKY rats fed a normal diet. However, the causal relationship between atherosclerosis and anomalous lipid metabolism has not been fully elucidated. It is also unclear how the development of hypertension and hypercholesterolemia may be related in SH rats.

In an effort to gain more insight into the mechanism by which increases in ChoE, particularly cholesterol oleate, occur, we have examined the effect of a high cholesterol diet on the fatty acid compositions of both hepatic and plasma lipids of SH rats. Aorta lipids were also analyzed to see the effects of the diet-induced changes in hepatic and plasma lipids on this tissue. We have also analyzed the molecular species of ChoE produced by plasma LCAT to estimate roughly the contribution of LCAT in the synthesis of plasma ChoE.

MATERIALS AND METHODS

SH rats (male, five wk of age, Charles River of Japan, Kanagawa, Japan) were fed either a conventional diet MF (Oriental Yeast Co., Tokyo, Japan) or a high cholesterol diet supplemented with 5% cholesterol and 0.5% cholate for up to 65 days. The diet MF contained by weight 24% protein, 5.1% fat, 6.2% minerals, 3.2% fibers and 54.5% non-nitrogenous compounds, with vitamins D_3 and K_3 supplemented. The major fatty acid constituents were linoleate (50%), oleate (22%), palmitate (16%), linolenate (4%) and stearate (2%). Tail systolic blood pressure was measured by the plethysmographic tail method with an apparatus produced by Natsume Co. (Tokyo, Japan).

After fasting overnight at the indicated days of test diets, three rats for each group were killed by decapitation, and livers, blood samples, abdominal and thoracic aortas were removed. Livers and plasmas from the blood samples were kept frozen at −80 C. Aortas were freed of surrounding fat tissue under the microscope and then kept frozen at −80 C. Lipids were extracted with chloroform/methanol according to the method of Bligh and Dyer (48). Neutral lipids and phospholipids were separated by chromatography on silica gel thin layer plates (Merck 60) which were prewashed with the developing solvents (petroleum ether/diethyl ether/acetic acid [80:30:1, v/v/v] or chloroform/methanol/water [70:30:5, v/v/v], respectively). The fatty acid composition of esterified lipids was determined by gas chromatography (GC) of fatty acid methyl esters using heptadecanoic acid as the internal standard. The amounts of lipids were expressed as mg of heptadecanoic acid per g of wet wt or per dl of plasma. Cholesterol was quantitated by GC of its trimethylsilyl ether using ergosterol as the internal standard. Phosphatidylcholine (PC) was hydrolyzed by phospholipase A_2 (*Crotalus adamanteus* venom) as described elsewhere (49) to identify and quantitate the fatty acids at the 1- and 2-positions separately. The lecithin:cholesterol acyltransferase (LCAT) assay was based essentially on the methods of Stokke and Norum (50) and Albers et al. (51), the details of which are described elsewhere (52). Briefly, plasmas kept frozen at −80 C were thawed and preincubated with a sulfhydryl reagent to inhibit LCAT activity, and then preincubated with [^{3}H]cholesterol (Amersham, Buckinghamshire, England) to equilibrate with endogenous cholesterol. The reaction was initiated by adding excess 2-mercaptoethanol to reactivate the LCAT. Molecular species of ChoE

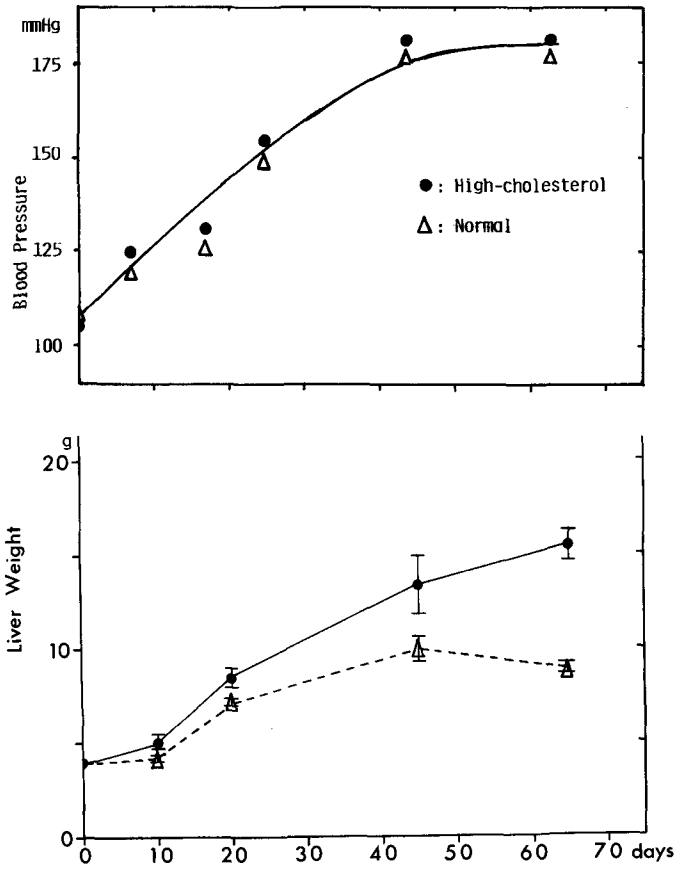

FIG. 1. Effect of a high cholesterol diet on blood pressure and liver weight. Rats (SH, five wk of age, male) were fed either a normal or a high cholesterol diet for the indicated periods. Tail systolic blood pressure was measured by the plethysmographic tail method. Each point represents an average of determinations for three rats (± S.E.).

synthesized by LCAT were determined by $AgNO_3$-silica gel thin layer chromatography.

RESULTS

General observation. Male rats (SH, five wk of age) were fed either the high cholesterol diet or the normal diet for up to 65 days. The differences in the diets did not affect body weight, blood pressure or relative microsomal protein content of liver. Rats in both groups developed a typical hypertension with systolic blood pressures of 150 mm Hg and 185 mm Hg at day 25 and 45, respectively, of the test diets (Fig. 1). On the other hand, significant differences between the liver weights of rats in the two dietary groups were apparent by day 20; liver weights of rats fed the high cholesterol diet were 50% greater by day 65 than those of control rats fed the normal diet. Accompanying the increases in liver weight was a fading of liver color to that typical of fatty liver.

Hepatic and plasma lipid compositions. As shown in Figure 2, the levels of ChoE increased greatly in rats fed the high cholesterol diet. After feeding the high cholesterol diet to rats for 45 days there was a 200-fold increase in the liver ChoE content. Free cholesterol and triacylglycerol increased 1.5- to threefold and fourfold, respectively. The amounts of free fatty acid, phosphatidylcholine and phosphatidylethanolamine were relatively unchanged. We emphasize that liver weight per rat was increased significantly by feeding the high cholesterol diet (Fig. 1) and hence the increases in hepatic lipids per rat were quite pronounced.

The plasma lipid composition was different from that of liver. There is more free cholesterol than cholesterol ester (Fig. 2). The high cholesterol diet elevated plasma free cholesterol 1.8-fold at day 20. The increase in ChoE was slightly less (1.5-fold). Both cholesterol and ChoE in

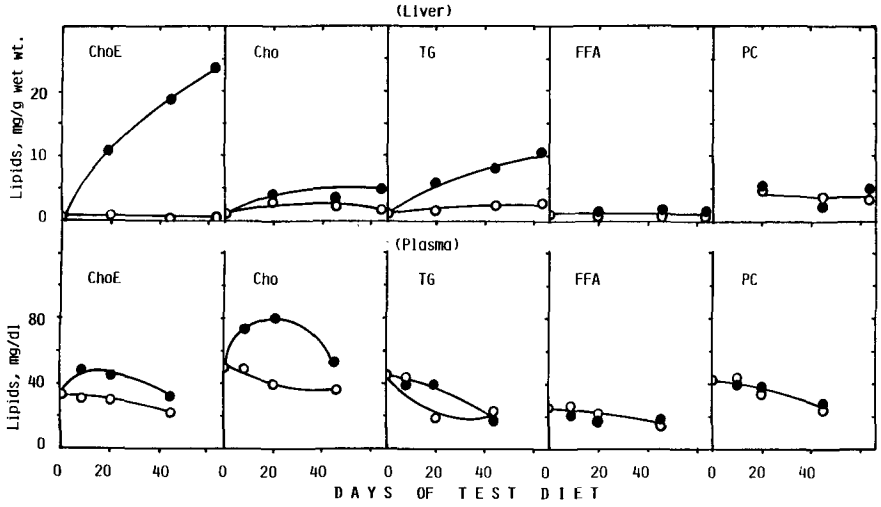

FIG. 2. Effect of a high cholesterol diet on hepatic and plasma lipid contents. After overnight fasting, three rats for each group were killed at the indicated days, samples of livers and plasmas were removed and lipids were extracted with chloroform/methanol. Individual lipid classes were separated by silica gel thin layer chromatography. Lipids containing fatty acids were quantitated by gas chromatography (GC) with heptadecanoic acid as internal standard. Cholesterol was quantitated as its trimethylsilyl ether by GC using ergosterol as internal standard. Each point represents average of determinations for at least two rats. The maximal deviation from mean was 24% of the value given. —•—, High cholesterol diet group; —○—, normal diet group.

plasma tended to decrease by day 45, regardless of the diet. Plasma triacylglycerol levels actually decreased after day 20 on the normal diet and after day 45 on the high cholesterol diet. The high cholesterol diet did not affect the total plasma free fatty acid or PC content, which tended to decrease after day 20. Thus, the 200-fold increase in liver ChoE and fourfold increase in liver triacylglycerol were not directly reflected by corresponding changes in plasma ChoE and triacylglycerol in the high cholesterol diet group, although the sizes of the ChoE pools in plasma and liver are comparable in rats fed the normal diet.

Fatty acid compositions of hepatic and plasma lipids. The fatty acid compositions of ChoE, free fatty acid, triacylglycerol and PC in liver from rats fed the test diets for 20 days are shown in Figure 3. Because the specificity of LCAT is related to the composition of fatty acids at the 2-position of PC (53–58), fatty acids at the 1- and 2-positions of PC were analyzed separately after phospholipase A_2 hydrolysis. As is well-known, each lipid exhibits a characteristic fatty acid pattern. The high cholesterol diet induced a striking change in the fatty acid composition of ChoE: the amounts of saturated and polyene fatty acids decreased while the quantities of oleate and linoleate increased 3.3- and 2.4-fold, respectively. Similar but slightly less pronounced changes were observed in the free fatty acid fraction. Nevertheless, there were only small changes in the fatty acid compositions of triacylglycerol, PC and phosphatidylethanolamine.

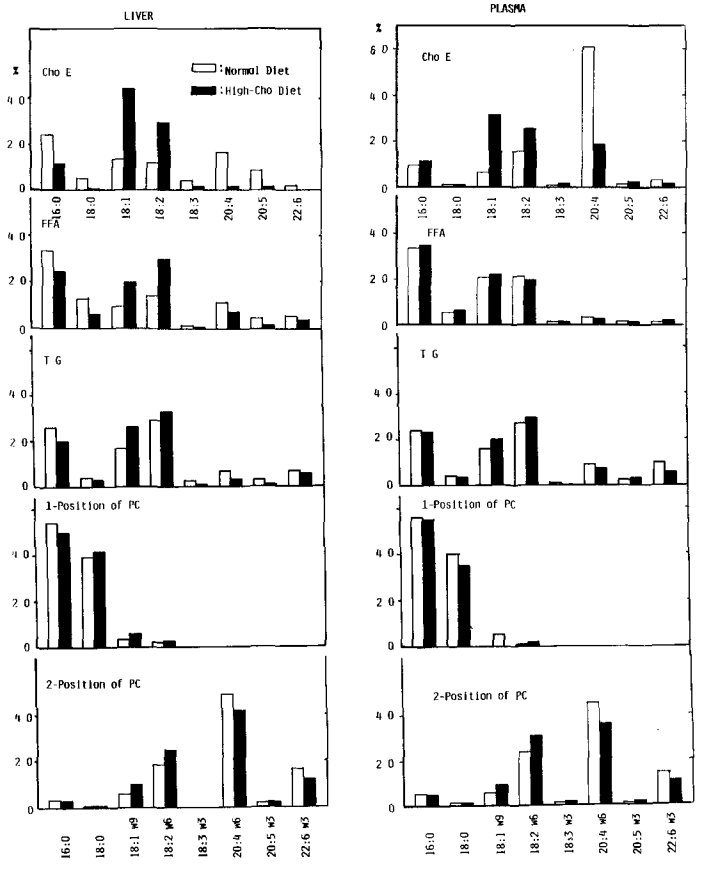

FIG. 3. Effect of a high cholesterol diet on fatty acid pattern of hepatic and plasma lipids. Rats fed test diets for 20 days were used. Fatty acids of hepatic and plasma lipids were analyzed by gas chromatography as methyl esters. Phosphatidylcholine (PC) was hydrolyzed with snake venom phospholipase A_2 to determine the fatty acids at the 1- and 2-positions, separately. Fatty acids were designated by carbon chain length:number of double bonds, and the position of double bond (indicated as ω3 [w3] or ω6 [w6]). Averages of values from two rats are presented. The maximum deviations from the means were 26 and 31% of the values when they were over 10% and 5% of the total, respectively.

The fatty acid compositions of plasma lipids are also shown in Figure 3. In comparing the fatty acid patterns of hepatic and plasma lipids, the following features should be noted: (i) In the normal diet group, plasma ChoE had fatty acid moieties quite different from those of plasma free fatty acid and hepatic ChoE; the plasma ChoE contained relatively more arachidonate and relatively less palmitate and ω3 fatty acids than hepatic ChoE; and the pattern of plasma ChoE fatty acids more closely resembled that at the 2-position of PC. (ii) In the high cholesterol diet group, the fatty acid pattern of plasma ChoE was more similar to that of hepatic ChoE and more unlike that of the 2-position of PC. The fatty acid composition of plasma ChoE differed significantly from that of the normal diet group; oleate and linoleate were increased and arachidonate was decreased by feeding the high cholesterol diet. (iii) The fatty acid compositions of plasma and hepatic PC were quite similar in spite of differences in diets (although the high cholesterol diet did cause a slight increase in oleate and linoleate and a concomitant decrease in arachidonate).

Comparisons of the fatty acids of hepatic and plasma lipids described above were made using livers and plasmas from the rats fed the test diets for 20 days. The time courses for the changes in the fatty acid compositions of hepatic ChoE are shown in Figure 4. The changes in the fatty acid compositions of hepatic ChoE induced by different diets were almost maximal by 20 days of feeding. There were significant decreases in saturated fatty acids and increases in ω9 fatty acids. The positions of double bonds in fatty acids have not been determined, and hence oleate described here represents octadecenoic acids (oleic and *cis*-vaccenic acids). Omega-9, ω6 and ω3 correspond to n-9, n-6 and n-3 series. The proportion of ω6 fatty acids as a whole did not vary significantly, although there were increases in linoleate and corresponding decreases in arachidonate induced by the high cholesterol diet.

The changes in the fatty acids of plasma ChoE were almost maximal after 20 days on the diets (Fig. 4). In fact, the maximum effect was observed even at day 9 of the diet. In contrast to the situation with hepatic ChoE, the proportions of saturated and ω3 fatty acids of plasma ChoE did not decrease but the proportion of ω6 fatty acids decreased.

Since the fatty acid composition of plasma PC was changed by feeding the high cholesterol diet, the molecular species of ChoE produced by plasma LCAT would also be expected to vary. To determine the extent of this variation, the molecular species of ChoE produced by plasma LCAT were analyzed in an in vitro assay system presumed to mimic that occurring in vivo (50–52). As shown in Figure 5, the LCAT showed some preference for arachidonate and less preference for linoleate at the 2-position of PC, but newly formed ChoE roughly reflected the changes in the fatty acid compositions of

PC induced by feeding different diets; the proportions of monoene and diene species increased and that of tetraene decreased by feeding the high cholesterol diet. However, these variations were much smaller than the diet-induced variations in the molecular species of plasma ChoE (see Discussion).

Aorta lipids. Lipids of the abdominal and thoracic aortas at day 20 and day 45 of the test diets were determined. The difference of the diets did not affect the amounts of PC (~1.4 mg/g). The triacylglycerol content was slightly higher in the high cholesterol diet group at day 45 (~2.6 mg/g) than in the control group (~1.5 mg/g). No accumulations of ChoE (~0.05 mg/g) and cholesterol (~1.2 mg/g) were observed in the aorta. The major fatty acids of PC were palmitate (39%), stearate (21%), oleate (11%), linoleate (4%) and arachidonate (18%) while those of triacylglycerol were palmitate (33%), palmitoleate (9%), stearate (5%), oleate (26%) and linoleate (19%). Again, the high cholesterol diet did not induce any significant changes in the fatty acid compositions of these lipids in aortas under the conditions examined.

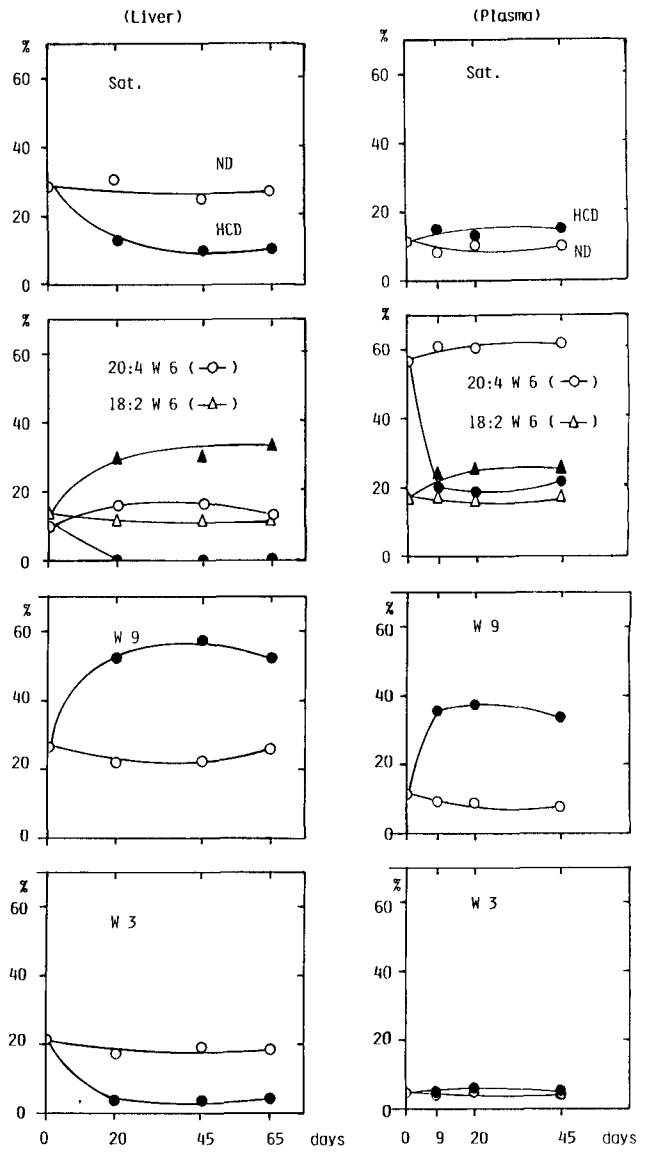

FIG. 4. Time course of the effect of a high cholesterol diet on fatty acid pattern of hepatic and plasma cholesterol ester. Fatty acids of ChoE were grouped into saturated (Sat., mainly 16:0 and 18:0), ω6 (linoleate, 18:2w6, and arachidonate, 20:4w6), ω9 (w9 and w7, mainly 18:1) and ω3 (w3, mainly 18:3w3, 20:5w3 and 22:6w3). ○, △, Normal diet group; •, ▲, high cholesterol diet group.

DISCUSSION

ChoE is thought to be formed mainly through the actions of LCAT in plasma and ACAT in liver and intestine. Since the rats in the present experiment were fasted overnight before plasma and liver samples were removed, the contribution of intestinal ACAT is probably relatively small under these conditions. LCAT utilizes fatty acids from the 2-position of PC, producing only unsaturated ChoE. ACAT is reported to be specific for monoene, saturated and diene species of acyl-CoAs (34,59-62). In the rats fed the normal diet, plasma ChoE consisted mainly of tetraene (arachidonate) species (60% of the total). Monoene (30%) and diene (26%) ChoE were the major species of plasma ChoE in rats fed the high cholesterol diet (Fig. 3). Since plasma LCAT synthesized mainly tetraene species regardless of the diet (Fig. 5), we simply surmised that plasma ChoE was synthesized mainly by LCAT in the normal diet group while the contribution of ACAT increased in the high cholesterol diet group. However, this needs to be examined more carefully.

Plasma free cholesterol, ChoE and triacylglycerol levels were elevated by the high cholesterol diet, but these

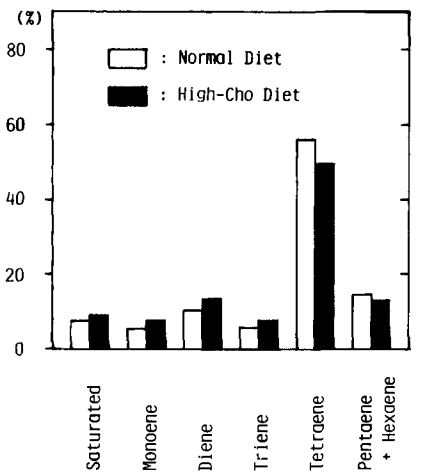

FIG. 5. Selectivity of plasma lecithin:cholesterol acyltransferase. Plasma samples from rats at 20 days of test diet were preincubated with an LCAT inhibitor, 5,5'-dithiobis(2-nitrobenzoic acid), and then with [^{3}H]cholesterol. Incubation was initiated by adding excess 2-mercaptoethanol to reactivate the LCAT. Molecular species of ChoE synthesized from [^{3}H]cholesterol and endogenous acyl donor (PC) were separated by $AgNO_3$-silica gel thin layer chromatography. Radioactivity in each spot was determined by liquid scintillation spectrometry. Although the LCAT inhibitor was not present in blood during the preparation of plasma, the amount of PC used by the LCAT reaction during this period is calculated to be much less than 0.5% of the total. Averages of values for two rats, each assayed in duplicate, are presented. The maximal deviations from means were 6% of the values given for saturated, monoene, diene and tetraene species and 20% for the pentaene plus hexaene species.

changes did not reflect directly the large increases in hepatic lipids. These findings suggest that a step after the synthesis and prior to secretion of ChoE and triacylglycerol is limited in the livers of rats fed the high cholesterol diet. The relative increase in linoleate with a concomitant decrease of arachidonate in ChoE raises the possibility that the elongation and/or desaturation systems became impaired during the development of fatty liver. On the other hand, the ω6 and ω3 fatty acid esters of cholesterol in liver and plasma responded differently to the dietary conditions (Fig. 4). Thus, the increase in ω9 fatty acids and the corresponding decrease in the proportions of polyene fatty acids must not be the only mechanism responsible for the changes in the fatty acid pattern of lipids under these conditions.

Although cholesterol oleate is the major lipid in human atherosclerotic lesions and the levels of hepatic and plasma cholesterol oleate increased significantly, no accumulation of cholesterol oleate was observed in the abdominal and thoracic aortas of SH rats fed a high cholesterol diet (5% cholesterol and 0.5% cholic acid) for up to 45 days. Our preliminary experiments revealed that plasma ChoE was even higher in normotensive WKY rats than in SH rats when the high cholesterol diet was fed for 45 days. On the other hand, in a substrain selected from SH rats, arteriolipidosis-prone (AL) rats, the plasma cholesterol level was reported to reach 500 mg/dl by feeding a high fat/cholesterol diet (20% suet, 5% cholesterol, 2% cholic acid) for one week (63). Fat deposits were noted in arteries but no significant difference was observed in the hepatic cholesterol levels in AL rats and normotensive controls under such conditions (63). Whether the difference in fat deposits in arteries is due to the difference in strains or the difference in diets is to be examined.

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