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Dietary 5-Campestenone (Campest-5-en-3-one) Enhances Fatty Acid Oxidation in Perfused Rat Liver

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Summary The effect of dietary 5-campestenone (campest-5-en-3-one), a chemical modification product of a naturally-occurring plant sterol, campesterol, on lipid metabolism was examined using a rat liver perfusion system. Male Sprague-Dawley rats weighing about 140 g were fed a diet supplemented with or without 0.2% 5-campestenone for 14 d. 5-Campestenone feeding resulted in a marked reduction in the concentrations of serum lipids, such as triacylglycerol (TG), cholesterol, phospholipid, and free fatty acid, without influencing food intake or growth. Then, isolated livers from both groups were perfused for 4 h in the presence of an exogenous linoelaidic acid substrate. Dietary 5-campestenone markedly elevated hepatic ketone body production, while cumulative secretions of TG, cholesterol, and phospholipid by the livers of rats fed 5-campestenone were all significantly lowered as compared to those fed without the compound; the extent of the reduction was more prominent in the secretion of TG than other lipid components. In addition, the reduction of TG secretion was concomitantly accompanied by the reduced incorporation of both exogenous and endogenous fatty acids into this lipid molecule. These results suggest that dietary 5-campestenone exerts its hypotriglyceridemic effect, at least, in part through an enhanced metabolism of endogenous and exogenous fatty acids to oxidation at the expense of esterification in rat liver.

Key Words 5-campestenone, perfused rat liver, ketone body production, triacylglycerol (TG) secretion

Epidemiological as well as experimental studies in animals have shown that there is a positive correlation between incidence of cardiovascular diseases and hyperlipidemia, and thus various types of drugs, such as pravastatin and simvastatin, and HMG-CoA reductase inhibitors, have been developed for the treatments of these hyperlipidemic patients (*1*).

It has long been recognized that the naturally-occurring plant sterols such as β-sitosterol, campesterol and stigmasterol exert a potent hypocholesterolemic activity through the interference with intestinal absorption of exogenous cholesterol in humans and experimental animals (*2–5*). On the other hand, it has been attempted to modify chemically the naturally-occurring sterols to strengthen their hypocholesterolemic activity. Several years ago, cholestanol, a microbial hydrogenated-derivative of cholesterol in the intestinal tract, was shown to exert a hypocholesterolemic activity in rats (*6, 7*), although this compound caused a detrimental side-effect, such as liver enlargement and pallor (*7*). After this finding, Sugano et al. found that phytostanol, especially β-sitostanol, a chemical hydrogenation product of phytosterol, exhibits a potent hypocholesterolemic activity much greater than original phytosterol, through an inhibitory effect on endogenous and exogenous cholesterol absorption in the intestine (*8–12*). These observations suggest that the hydrogenation of the steroid ring at the specific position plays an important role in inhibiting absorption of cholesterol, leading to a hypocholesterolemic activity of stanols.

On the other hand, Suzuki et al. recently reported that cholestenone (cholest-4-en-3-one), another bacterial metabolite of cholesterol in the intestine, is capable of lowering the concentration of serum triacylglycerol (TG) and reducing the accumulation of body fats, in addition to its hypocholesterolemic activity (*13, 14*). Others also reported similar results (*15, 16*), suggesting that 3-oxo modification of the 3-hydroxy group of the steroid ring is essential to cause beneficial effects on lipid metabolism.

Based on these findings, Suzuki et al. examined the chemical modification of plant sterols, such as campesterol and β-sitosterol to cause more potent effects on lipid metabolism, and found that 5-campestenone (campest-5-en-3-one, a 3-oxo derivative of campesterol) is the most effective and safe compound for improvement of hyperlipidemia (*17, 18*). They also recently

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found that this compound also acts as an anti-diabetic and anti-obesity, in addition to hypolipidemic actions (*19, 20*), although the mechanism(s) responsible for these beneficial effects remains obscure. In this study, we focused on elucidating the mechanism responsible for the hypotriglyceridemic effect of 5-campestenone using a rat liver perfusion system, and investigated whether this effect is, in part, due to an altered hepatic metabolism of fatty acids between the pathways of oxidation and esterification.

MATERIALS AND METHODS

Materials. Bovine serum albumin Fraction V and β-hydroxybutyrate dehydrogenase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Linoelaidic acid (*trans,trans*-9,12-octadecadienoic acid) was obtained from Sigma Chemical (St. Louis, MO, USA) and Cayman Chemical (Ann Arbor, MI, USA). Pentadecanoic acid was obtained from Sigma Chemical. 5-Campestenone was supplied by RIKEN (Tecnoflora Co., Saitama, Japan).

Animals and diets. Male Sprague-Dawley rats weighing 90–100 g (4-wk-old) obtained from a local breeder (Seac Yoshitomi Ltd., Fukuoka, Japan) were housed individually in a room with controlled temperature (22–24°C), humidity (55–65%) and lighting (lights on 7:00–19:00), and maintained on a powdered commercial chow (Type CE-2, CLEA Japan, Inc., Tokyo, Japan) ad libitum and water. After acclimatization for 7 d, the rats weighing about 140 g were divided into two groups with equal body weights. The experimental diets were prepared according to the recommendations of the American Institute of Nutrition (*21*), the control diet was as follows (in weight%): casein, 20; corn oil, 5; mineral mixture (AIN 76), 3.5; vitamin mixture (AIN 76), 1; DL-methionine, 0.3; choline bitartrate, 0.2; cellulose, 5; β-corn starch, 15; and sucrose to 100. For the 5-campestenone diet, this compound at a level of 0.2% was added at the expense of sucrose. The animals had free access to each diet and deionized water for 14 d. Food intake and body weight were recorded every other day.

Liver perfusion experiment. On the day of perfusion experiments, rats were given an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight) at around 9:00. Blood was withdrawn from the portal vein just before insertion of a glass cannula into the portal vein, and followed by centrifugation at 1,000×*g* for 20 min to obtain the serum; it was stored at −80°C until lipid analyses were performed.

The apparatus for rat liver perfusion is shown in the Fig. 1. The livers were then isolated by the following procedure (The number and letter within the parenthesis correspond to those in the Fig. 1): the bile duct was cannulated with a polyethylene tubing and the bile was collected to confirm the bile flow was constant (I); the hepatic portal vein was cannulated with a glass cannula (II); the superior vena cava was cannulated with a glass cannula (III); the isolated liver was suspended from the ceiling of the cabinet (g); and the liver was placed on a platform (f). The temperature in the cabinet (g) was maintained with a light bulb (h). And then, the livers were perfused with Krebs-Henseleit buffer (pH 7.4) containing 1.5% (w/v) bovine serum albumin and 25% (v/v) washed bovine erythrocytes in a water-jacked cylindrical Plexiglas reservoir to maintain the perfusate at a constant temperature (a) at the rate of 20 mL/min by a peristaltic pump which maintains a uniform and nonpulsating flow (b). The perfusate was flowed to a silastic tubing lung (*22*) continuously equilibrated with 95% O_2 and 5% CO_2 (c), a filter (d), a bubble trap (e), the hepatic portal vein (*2*), the liver, the superior vena cava (*3*), and returned to a mixing chamber (a) at the place of (A). At the beginning of recirculation, 5 mL of 20 mM potassium linoelaidic acid (100 μmol) as the exogenous fatty acid substrate was added into the perfusate directly at the place of (A) and the same solution was infused continuously into (A) at the

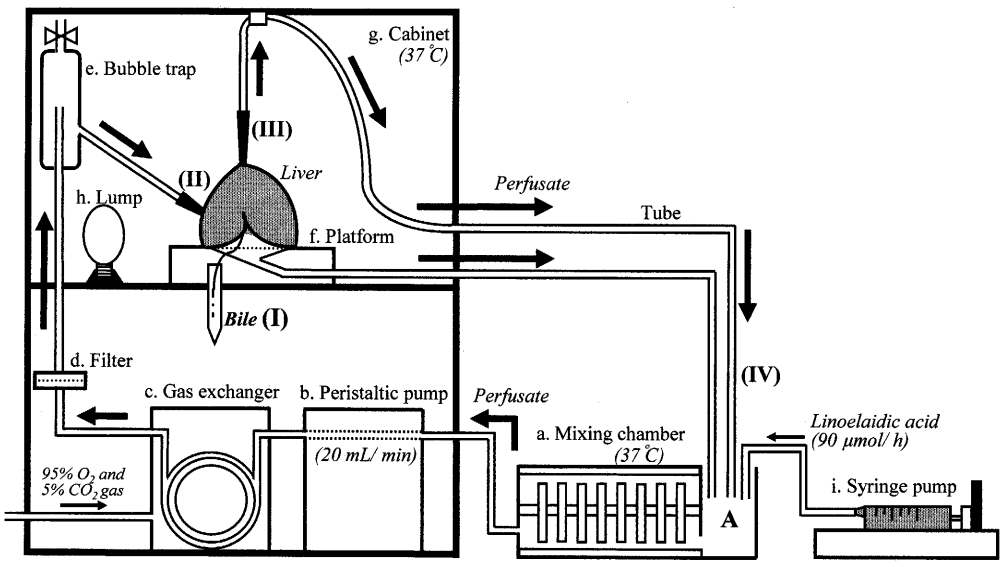

Fig. 1. Apparatus for rat liver perfusion.

rate of 4.5 mL/h (90 μmol/h) by a mechanical infusion pump (i). About 20 mL of perfusion was removed from the tube (IV) for analyses of ketone bodies and lipids every 1 h, and the same amount of fresh perfusion medium was added into (A) at each removal to maintain a recirculation volume of 120 mL. The liver perfusions of the control and 5-campestenone groups were performed at the same time with different liver perfusion apparatus, and continued for a total of 4 h. The perfusion equipment and procedure were those described in detail previously, and liver appearance and rates of perfusate and bile flow were used as indices of liver function, in addition to the β-hydroxybutyrate; acetoacetate ratio and the rates of TG secretion and ketogenesis were constant (*23*). The experimental protocol was approved by the Ethics Committee for Animal Experiments of University of Miyazaki.

After the end of perfusion, total perfusates were collected and centrifuged to remove erythrocytes, and then the ketone bodies were measured immediately. Post-perfused livers were rinsed, weighed and homogenized with cold saline solution. The remainders of perfusates and liver homogenates were stored at −80°C until lipid analyses were performed.

Analyses of ketone bodies and lipids. Serum TG, total cholesterol, phospholipid, and free fatty acid concentrations were determined enzymatically using available commercial kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). β-Hydroxybutyrate and acetoacetate were measured enzymatically in deproteinized sample of erythrocyte-free perfusate as described previously (*23–25*). The lipids from erythrocyte-free perfusate and post-perfused liver were extracted and purified according to the method of Folch et al. (*26*), and followed by measurement of TG, cholesterol, and phospholipid. TG in perfusate at the end of perfusion and post-perfused liver were separated by silica gel 60G thin-layer chromatography with a solvent mixture of *n*-hexane : diethyl ether : glacial acetic acid (80 : 20 : 1, v/v/v), and followed by transesterification of fatty acids with methanolic H_2SO_4, and the fatty acid composition analyzed by gas-liquid chromatography (GC-14A and-12A, Simadzu Co., Kyoto, Japan) on a Supelcowax-10 column with temperature rising (*27*). The free fatty acid composition of the perfusate at 1 h intervals was also analyzed with gas-liquid chromatography after separation with thin-layer chromatography as described above. The free fatty acid concentration was calculated using pentadecanoic acid as the internal calibration standard.

Statistical analysis. Data were expressed as mean±SE, and the statistical significance of the difference in the means was evaluated by Student's *t*-test at the level of $p<0.05$ (*28*).

RESULTS

Growth, food intake, liver weight, and lipid parameters in serum and post-perfused liver (Table 1)

There were no significant differences between the different groups with respect to food intake, final body weight, or relative liver weight after perfusion. Dietary 5-campestenone markedly reduced the serum lipid concentrations; the extents of the reduction were 52% in TG, 30% in cholesterol, 27% in phospholipid, and 34% in free fatty acid compared with control group. In the post-perfused liver, TG concentration tended to decrease by 40%, although the difference was not statistically significant due to large variations of the two groups. Hepatic cholesterol concentration was also significantly lowered in the 5-campestenone-fed rats, while phospholipid remained unchanged.

Table 1. Effect of 5-campestenone feeding on body weight, food intake, liver weight, and lipid concentrations in serum and liver.

	Control	5-Campestenone
Growth parameters		
Initial BW (g)	140±5	147±6
Final BW (g)	276±6	286±11
Food intake (g/d)	22.7±0.4	22.9±0.6
Liver weight (g/100 g BW)	6.72±0.27	7.12±0.23
Serum lipid concentrations		
Triacylglycerol (mg/dL)	207±28	99±12**
Total cholesterol (mg/dL)	150±12	105±6*
Phospholipid (mg/dL)	279±13	204±1**
Free fatty acid (μEq/dL)	115±6	77±6**
Post-perfused liver lipid concentrations (μmol/g)		
Triacylglycerol	136±25	82.5±17.0
Cholesterol		
Total	10.2±0.6	6.15±0.20***
Free	5.34±0.19	4.60±0.06**
Ester (%)	46.5±3.5	24.8±2.3***
Phospholipid	28.4±1.4	32.6±1.7

Values are expressed as mean±SE of seven rats.
Significantly different from control group at * $p<0.05$, ** $p<0.005$ and *** $p<0.0005$.
Rats weighing about 140 g were fed the 20% casein diet with or without 0.2% 5-campestenone for 14 d. The serum was obtained from blood in the portal vein. The livers were isolated and perfused for 4 h in the presence of exogenous linoelaidic acid substrate. Liver weight was measured at the end of perfusion.
BW, body weight.

Hepatic uptake of free fatty acid substrates, which was calculated from the amounts infused and the amounts of free fatty acid present in the perfusate at the consecutive time intervals, was relatively constant throughout the perfusion period, and the cumulative extraction for a total of 4 h perfusion periods was statistically similar in the two groups; 9.3±0.5, 14.1±0.7, 18.5±0.9 and 21.5±0.9 μmol/g liver for control group, and 9.2±0.4, 13.6±0.6, 17.7±0.8 and 20.9±1.0 μmol/g liver for 5-campestenone group. Therefore, the following events on the diverse responses in ketone body production and lipid secretion by the perfused livers from rats fed diets containing 5-campestenone as compared with the control diet could most exclusively be attributed to the influence of the compound per se. (*23–25*).

Table 2. Effect of 5-campestenone feeding on cumulative production of ketone bodies and the ratio of β-hydroxybutyrate to acetoacetate.

	Perfusion period (h)			
	1	2	3	4
Ketone body production (μmol/g liver)				
Control	12.6±2.0	17.9±3.6	24.0±4.9	28.8±5.6
5-Campestenone	21.6±2.4*	33.3±4.6*	43.4±6.4*	52.5±6.8*
β-Hydroxybutyrate/acetoacetate ratio				
Control	0.66±0.06	0.76±0.04	0.83±0.06	1.10±0.15
5-Campestenone	0.63±0.07	0.68±0.09	0.84±0.14	1.13±0.20

Values are expressed as mean±SE of seven rats.
* Significantly different from control group at $p<0.05$.
Experimental conditions were the same as those described in legends to Table 1.

Table 3. Effect of 5-campestenone feeding on cumulative secretion of triacylglycerol, cholesterol, and phospholipid by perfused liver.

	Perfusion period (h)			
	1	2	3	4
Triacylglycerol secretion (μmol/g liver)				
Control	0.50±0.05	1.19±0.09	2.29±0.13	3.03±0.20
5-Campestenone	0.31±0.05*	0.70±0.14*	1.08±0.25**	1.35±0.32**
Cholesterol secretion (μmol/g liver)				
Control	0.17±0.02	0.23±0.02	0.33±0.02	0.44±0.05
5-Campestenone	0.12±0.02	0.16±0.02*	0.24±0.03*	0.29±0.03*
Phospholipid secretion (μmol/g liver)				
Control	0.29±0.07	0.59±0.08	0.91±0.12	1.36±0.18
5-Campestenone	0.14±0.03	0.32±0.07*	0.50±0.10*	0.88±0.13

Values are expressed as mean±SE of seven rats.
Significantly different from control group at * $p<0.05$ and ** $p<0.005$.
Experimental conditions were the same as those described in legends to Table 1.

Ketone body production and lipid secretion by the liver

Table 2 shows the time-course of ketone body production and the ratio of β-hydroxybutyrate to acetoacetate. The production of ketone bodies by the perfused livers was approximately linear during the perfusion period in each group, indicating that livers functioned normally for 4 h of perfusion periods. Ketone body production, a marker of enhancement of fatty acid oxidation, was elevated to approximately 2 fold at the end of perfusion in the 5-campestenone-fed group as compared to the control group. On the other hand, the ratio of β-hydroxybutyrate to acetoacetate, which is an indication of mitochondrial redox potential (*29, 30*), was not significantly different between the two groups.

The cumulative secretions of lipids by the livers are shown in Table 3. 5-Campestenone feeding resulted in a marked decrease in hepatic secretion of TG at all time points; the extent of reduction at the end of perfusion was 55% compared with control group. Hepatic secretions of cholesterol and phospholipid were also significantly decreased; the extents of reduction of these two lipid components at the end of perfusion were similar (35% reduction), although the extents of these reductions were less than that of TG.

Fatty acid composition of perfusate and liver TG

Table 4 summarizes the fatty acid composition of TG in perfusate obtained at the end of perfusion and post-perfused liver. In this study, we used linoelaidic acid as an exogenous fatty acid because this di-*trans* fatty acid is not synthesized within an organism and is easily detected as an exogenous fatty acid in distinction from endogenous fatty acids by gas-liquid chromatography (*31, 32*), although there are subtle differences in the metabolic fate of *trans*-fatty acids as compared to *cis*-counterparts with respect to their oxidation and esterification (*33, 34*). In the control group, the percentages of exogenous linoelaidic acid were 17.3 and 1.2% in perfusate and post-perfused liver, respectively, indicating that the fatty acid supplied exogenously is incorporated into hepatic TG and is actively secreted in the form of TG-fatty acids. On the other hand, in the 5-campestenone group, the proportions of exogenous di-*trans* fatty acid were found to be 5.8 and 0.6% in the perfusate and liver, respectively and were significantly low-

Table 4. Fatty acid composition and calculated amounts of endogenous and exogenous *trans* fatty acids of triacylglycerol in perfusate and post-perfused liver.

	Fatty acid					
	16:0	16:1	18:0	18:1	18:2	
					cis,cis	*trans,trans*
Perfusate triacylglycerol (mol%)						
Control	27.2±1.7	6.9±0.5	2.1±0.1	39.6±1.5	7.0±0.7	17.3±2.0
Campestenone	28.1±1.1	4.3±0.4**	2.3±0.1	56.1±1.7***	3.3±0.4***	5.8±0.5***
Post-perfused liver triacylglycerol (mol%)						
Control	39.3±2.9	8.9±1.2	2.9±0.6	42.2±1.6	5.4±1.8	1.2±0.2
Campestenone	35.0±1.0	5.1±0.4**	2.5±0.1	53.9±1.5***	2.9±0.5	0.6±0.1**
Perfusate triacylglycerol (μmol/g liver)[1]						
Control	2.44±0.15	0.63±0.06	0.19±0.01	3.61±0.31	0.64±0.09	1.58±0.24
Campestenone	1.17±0.31**	0.18±0.05***	0.09±0.02***	2.22±0.51*	0.14±0.04***	0.24±0.06***
Post-perfused liver triacylglycerol (μmol/g liver)[2]						
Control	170±35	40.9±10.3	10.1±1.0	166±28	16.3±1.8	4.3±0.6
Campestenone	87.4±18.3	13.6±3.3*	6.0±1.2*	131±26	8.2±2.5*	1.4±0.2**

Values are expressed as mean±SE of seven rats.
Significantly different from control group at * $p<0.05$, ** $p<0.005$ and *** $p<0.0005$.
Experimental conditions were the same as those described in legends to Table 1.
[1] [μmol of triacylglycerol secreted during 4 h×3×percentage of endogenous or exogenous *trans* fatty acids in perfusate/100].
[2] [μmol of triacylglycerol in post-perfused liver×3×percentage of endogenous or exogenous *trans* fatty acids/100].

ered compared with control group. With respect to endogenous fatty acids, the percentages of linoleic and palmitoleic acids in the perfusate TG were also significantly lowered, while those of oleic acid increased significantly in the 5-campestenone group as compared to the control group. No significant differences were noted in the compositions of other endogenous fatty acids. The same responses were also observed in liver TG.

The amounts of individual fatty acids of TG secreted into perfusate and stored in the liver were calculated, based on the percentages of the fatty acids (Table 4). In perfusate TG, dietary 5-campestenone caused a marked decrease in the concentration of exogenous di-*trans* fatty acid (0.24± 0.06 vs. 1.58± 0.24 μmol/g liver). In addition, the concentrations of endogenous fatty acids were all significantly lowered in rats fed diets containing 5-campestenone as compared to those fed without the compound. On the other hand, the incorporation of exogenous and endogenous fatty acids into livers were also reduced in 5-campestenone group as compared to control group, but to a lesser extent than the changes observed in the perfusate TG.

DISCUSSION

It has been reported that 5-campestenone modified chemically from campesterol is a newly developed compound that exhibits hypolipidemic, anti-obese, and anti-diabetic efficacies in mice and rats (*18–20*). In the present study, 0.2% 5-campestenone feeding for 14 d caused a significant reduction in the concentration of serum lipids, such as TG, cholesterol, phospholipid, and free fatty acid in rats, consistent with previous observations by Suzuki et al. (*19, 20*) and Ikeda et al. (*35*). This hypolipidemic effect was observed with no undesirable effects on growth parameters. Ikeda et al. observed a reduced food intake when added at the level of 0.5% 5-campestenone to the diet (*35*). In the present study, dietary 5-campestenone at the level 0.2% did not show any undesirable effects on food intake and growth.

In the present studies, we investigated the mechanism underlying the hypotriglyceridemic effect of 5-campestenone using a liver perfusion experiment, since the mechanism(s) responsible for the observed reduction in the concentration of serum and liver lipids, especially TG, remained to be clarified. In this study, we have found that ketone body production by the livers of rats fed diets containing 5-campestenone as compared to those fed diets without the compound was significantly elevated, suggesting a stimulatory effect of dietary 5-campestenone on fatty acid oxidation, although it is unknown whether dietary 5-campestenone increases the blood ketone body concentration in vivo. On the other hand, the ratio of β-hydroxybutyrate to acetoacetate, which is an indication of mitochondrial redox potential (*29, 30*), did not differ between the groups. Ikeda et al. have recently reported that 5-campestenone feeding stimulated the activity of enzymes related to fatty acid oxidation such as mitochondrial carnitine palmitoyltransferase and peroxisomal β-oxidation in rat livers (*35*). Thus, these observations therefore suggest that 5-campestenone is a compound capable of enhancing the activities of enzymes related to fatty acid oxida-

tion in both mitochondria and peroxisomes. These responses in fatty acid oxidation are similar to those observed in rats fed sesamin, a sesame lignan (*32*), and fibrates (*36*), hypolipidemic drugs widely used in the world.

It has long been recognized that there is a reciprocal response in ketogenesis and TG secretion under various nutritional and physiological conditions (*23–25, 31, 32, 36, 37*). The present experiment evidently showed that the enhancement of ketone body production was inversely related to reduction of TG secretion by the liver. This result suggests that the inversed relationship of fatty acids between fatty acid oxidation and synthesis and secretion of TG is a major determinant for the hypotriglyceridemic activity of dietary 5-campestenone, consistent with previous experiments with the dietary sesamin and fibrates (*32, 36*).

It is known that fatty acids utilized for esterification, especially for the formation of TG, are derived from serum free fatty acids, de novo fatty acid synthesis, and the intrahepatic lipolytic process. The relative contribution of these fatty acid sources in the liver is variable under various physiological and nutritional conditions (*24, 25, 31*). In the present studies, we assessed the contribution of the exogenous fatty acid, using linoelaidic acid, as compared to that of endogenous sources, for the formation of TG in the liver. We have previously discussed the relevance of the linoelaidic acid in liver perfusion experiments to examine the fate of an exogenous fatty acid substrate (*31, 32*). In this study, the reduction in the secretion of TG by the liver was concomitantly accompanied by the decreased incorporation of exogenous linoelaidic acid in TG molecules. The incorporations into perfusate TG of endogenous fatty acids, probably derived from intrahepatic lipolytic events, were also significantly decreased; however, the relative contribution of exogenous linolelaidic acid was greater than that of endogenous fatty acids such as oleic and palmitic acids. These observations suggest that dietary 5-campestenone altered hepatic metabolism of fatty acids, particularly exogenous linoelaidic acid, between oxidation and esterification, leading to the hypotriglyceridemic activity of this compound in vivo in the rat. These responses are similar to those observed in rats fed sesamin and fibrates (*32, 36*).

In this study, dietary 5-campestenone also decreased hepatic secretion of cholesterol, accompanied by the reduced concentration of serum and liver cholesterol. It is known that plant sterols, such as campesterol, an original compound of 5-campestenone, exert a potent hypocholesterolemic activity through an inhibition of cholesterol absorption in the intestinal tract (*2–5*). Therefore, it can be speculated that hypocholesterolemic action of 5-campestenone, like plant sterols, may also exert itself through the interference with intestinal cholesterol absorption of cholesterol (*35*). It cannot be ruled out that 5-campestenone may suppress the biosynthesis of cholesterol (*15, 38*), although the mechanism(s) responsible for observed reduction in the hepatic secretion and serum and liver concentration of cholesterol remains to be determined.

In summary, we demonstrated for the first time that dietary 5-campestenone decreased synthesis and secretion of esterified lipids, especially TG, and the reduction was inversely related to the elevated hepatic fatty acid oxidation in perfused rat liver. These observed results along with a potential inhibition of intestinal cholesterol absorption can account for the hypolipidemic effect of 5-campestenone.

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