Apolipoprotein CIII Deficiency Prevents the Development of Hypertriglyceridemia in Streptozotocin-Induced Diabetic Mice

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To explore the role of apolipoprotein (apo) CIII in the development of hypertriglyceridemia associated with diabetes mellitus, we examined triglyceride (TG) kinetics in apo CIII – deficient mice (apo CIII – null) and wild-type (WT) (C57BL/6J) mice with diabetes induced by the injection of streptozotocin (STZ). Plasma TG levels increased significantly in WT mice after diabetes was induced (102 ± 29 *v* 65 ± 33 mg/dL, *P* < .01). Apo CIII–null mice had a significantly lower TG level (35 ± 9 mg/dL) that remained unchanged even when diabetes was induced (35 ± 8 mg/dL). The TG secretion rate (TGSR) measured by the Triton WR1339 method tended to decrease in diabetic WT, indicating that catabolism of TG was impaired. Apo CIII–null mice showed 2-fold higher TG production than WT mice, indicating markedly faster clearance of TG. The high TGSR was halved when diabetes was induced in apo CIII–null mice, and the fractional catabolic rate (FCR) of TG was also halved, although it was still significantly higher than in WT mice. Lipoprotein lipase (LPL) activity in postheparin plasma was not significantly altered in WT or apo CIII–null mice regardless of the presence or absence of diabetes. [^{3}H] very–low-density lipoprotein (VLDL)-TG from WT or apo CIII–null mice showed similar clearance by WT recipients, and this was also observed when VLDL was obtained from diabetic counterparts. In contrast, VLDL-TG was cleared faster by apo CIII–null recipients compared with WT recipients, regardless of the VLDL donors. These results suggest that apo CIII deficiency prevents the development of hypertriglyceridemia associated with diabetes by stimulating TG removal, possibly by promoting the interaction of VLDL with the TG removal system.

THE MOST COMMON plasma lipid abnormality in diabetic subjects is elevation of plasma triglyceride and reduction in high-density lipoprotein (HDL) cholesterol, which is believed to be associated with a high incidence of coronary heart disease in the diabetic population.[1] A number of experimental studies have attempted to elucidate the mechanism of hypertriglyceridemia associated with diabetes by using streptozotocin (STZ)-induced diabetic rats as the animal model.[2-8] It is well known that the hypertriglyceridemia in STZ-induced diabetic rats is solely due to a defect of triglyceride (TG) catabolism, because hepatic TG production is suppressed in the insulin-deficient state.[5,7,8] However, several studies have shown that lipoprotein lipase (LPL), a key enzyme for TG hydrolysis, is not markedly decreased in animals with STZ-induced diabetes.[8-10] Therefore, it is hypothesized that abnormalities of TG-rich lipoprotein particles are responsible for the defective catabolism of TG. Bar-On et al first reported that apolipoprotein (apo) CIII was increased and apo E was decreased in very–low-density lipoprotein (VLDL) obtained from STZ-induced diabetic rats,[2] and they suggested that these changes of lipoprotein particles were associated with delayed clearance from the plasma.[3,4] Recent gene-targeting studies performed in mice have revealed that apo CIII plays a primary role in raising the plasma TG level, since human apo CIII transgenic mice develop severe hypertriglyceridemia,[11-14] while apo CIII–deficient mice are hypotriglyceridemic and show resistance to a postprandial increase of plasma TG.[15] A number of clinical and experimental studies have demonstrated that apo CIII inhibits the action of LPL[14,16] and the hepatic uptake of TG-rich lipoprotein particles.[17] Conversely, apo E promotes the removal of TG-rich lipoprotein as a ligand for lipoprotein receptors, and this stimulates the uptake of lipoproteins by hepatocytes.[18,19] Therefore, it is reasonable to assume that an increase of apo CIII and a decrease of apo E in VLDL is a primary cause of the hypertiglyceridemia that occurs in STZ-induced diabetes as suggested by Bar-On et al.[3,4] However, it still remains unclear whether the increase of apo CIII or the decrease of apo E is more closely associated with hypertiglyceridemia. In addition, it is poorly understood whether lipoprotein abnormality or a defect of the TG removal system, such as lipolysis by LPL and organ uptake via lipoprotein receptors, is more deeply involved in the hypertiglyceridemia associated with STZ-induced diabetes.

To address these issues, we examined VLDL-TG kinetics in apo CIII–deficient mice (apo CIII–null) after diabetes was induced by the injection of STZ. We recently reported that nondiabetic apo CIII–null mice showed significant hypotriglyceridemia even though TG production was significantly stimulated,[20] indicating a substantial increase of TG removal. If an increase of apo CIII is important in the defective catabolism of TG in STZ-induced diabetic animals, the catabolic defect would not be detected in the diabetic mice lacking apo CIII.

MATERIALS AND METHODS

Mice

We used homozygous apo CIII–deficient mutants that were originally established by Maeda et al.[15] Apo CIII–null (C57 BL/6J background) and wild-type (WT) (C57 B/6J) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and the animals were mated in our laboratory. Only male mice were used to avoid the effect of sex differences on lipoprotein metabolism. STZ (100 mg/kg body weight) was injected intraperitoneally once a day for 5 days in mice aged 16 to 20 weeks according to the experimental design of Ebara et al,[21] and diabetic mice were used 2 weeks after the last injection of STZ. All mice were kept on a rotating 12-hour light/dark cycle and were given

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Table 1. Body Weight and Plasma Levels of Glucose and Total Cholesterol in WT and Apo CIII–Null Mice With and Without Diabetes

	WT		Apo CIII–Null	
	Nondiabetic	Diabetic	Nondiabetic	Diabetic
n	15	12	14	13
Body weight (g)	26.5 ± 2.2	23.8 ± 1.2*	27.2 ± 0.6	24.6 ± 1.2†
Plasma glucose (mg/dL)	148 ± 25	394 ± 35*	147 ± 32	364 ± 90†
Total cholesterol (mg/dL)	41 ± 16	50 ± 12	50 ± 24	39 ± 18

*$P < .01$, *v* nondiabetic WT.

†$P < .01$ *v* nondiabetic apo CIII–null.

free access to food and water. The mice were fed ad libitum with standard rat chow (Oriental Food Co, Tokyo, Japan), which contained 60% vegetable starch, 11% corn oil, and 29% animal protein. Food was withdrawn at 9 AM on the day of the experiments and all experiments were performed after a 5-hour fast. Animals were anesthetized with methoxyflurane for retro-orbital phlebotomy and for intravenous injection.

TG Secretion Rate

The TG secretion rate (TGSR) was determined by measuring the increase of the plasma TG concentration after an intravenous injection of Triton WR 1339 (Sigma Chemical Co, St Louis, MO) (500 mg/kg body weight, 25 % solution in saline). Mice were anesthetized with pentobarbital sodium (0.4 mg/100 g body weight, Nembutal, Dinabbot, Osaka, Japan), and blood was collected immediately before Triton WR 1339 injection and at 60 and 90 minutes afterwards. The plasma TG concentration was found to increase linearly over the 90-minute period in each mouse. TGSR was calculated from the increment of TG per minute multiplied by the plasma volume (estimated as 0.035% of body weight [g]) and was expressed as milligrams per minute. The validity of the Triton method for estimating TGSR has been described elsewhere.[8,23] The majority of TG (>90%) was recovered in the VLDL fraction (density < 1.006 g/mL) of both pre- and post-Triton plasma, so the TGSR determined by the Triton WR 1339 method is virtually equal to the rate of hepatic VLDL-TG secretion. The fractional catabolic rate (FCR) was calculated as TGSR divided by the TG pool size, as described previously.[8,20,22]

VLDL Clearance

The clearance of VLDL was studied by injecting radiolabeled VLDL-TG and monitoring the decrease of plasma radioactivity. The protocol for radiolabeling of VLDL-TG has been described in detail elsewhere.[24,25] Briefly, VLDL was endogenously radiolabeled with 400 μCi of [^{3}H]-glycerol (New England Nuclear, Boston, MA) per mouse. Plasma was collected from donor mice of the same genotype (WT or apo CIII–null) that received the same treatment (nondiabetic or diabetic). Five to 6 WT mice and 10 to 12 apo CIII–null mice, respectively, were needed to obtain a high VLDL radioactivity. VLDL (density < 1.006 g/mL) was separated from the pooled plasma by ultracentrifugation (Hitachi CP-65G; Hitachi Co, Tokyo, Japan) using an RP 55T-708 rotor (Hitachi). Then [^{3}H]-VLDL-TG (0.2 mg as TG) was reinjected into pentobarbital-anesthetized recipients. We used nondiabetic WT or apo CIII mice as recipients for the re-injection study. Blood samples (100 μL each) were collected from the retro-orbital plexus at 1, 3, 5, 7, and 9 minutes after the injection of VLDL. Plasma TG was extracted with 2-propanol and Zeolite mixture (Sigma) to completely remove the radioactivity of free glycerol.[24] We previously showed that, under these conditions, more than 97% of the label in the isopropyl alcohol was in TG.[7,8] A curve showing the decrease of circulating [^{3}H]-VLDL-TG in the recipient mice was drawn to indicate the percent of residual radioactivity when the radioactivity at 1 minute was 100%. It was difficult to calculate the FCR of TG in individual animals because the radioactivity at the indicated times varied significantly and the decay curve did not well fit for monoexponential or multiple compartment kinetics. Therefore, we simply determined the percentage of residual radioactivity at the indicated times after injection of [^{3}H]VLDL-TG.

LPL in Postheparin Plasma

Postheparin blood samples were obtained from 10 minutes after the injection of heparin (100 U/kg) into the femoral vein, and LPL activity was determined as the rate of release of radiolabeled fatty acids from a [^{14}C]triolein emulsion in Triton X 100 according to the method of Krauss et al.[26]

Measurements

The plasma TG concentration was determined by the enzymatic method using a commercially available kit (Triglyceride-G test, Wako Pure Pharmaceutical Co, Osaka, Japan). Plasma glucose levels were determined by the glucose oxidase method (Glucose B-test, Wako).

Data are expressed as the mean ± SD. Statistical significance was assessed by unpaired *t* test for 2 groups and 1-way analysis of variance (ANOVA) for more than 2 groups; and *P* values less than .05 were accepted as significant.

RESULTS

General Profile

Table 1 shows the body weight and the plasma levels of total cholesterol and glucose. STZ-induced diabetes caused similar weight loss in WT and apo CIII–null mice and similar marked hyperglycemia also developed in both types of mice. The total cholesterol level was comparable between WT and apo CIII–null mice and the levels was not altered when diabetes was induced.

TG Kinetics

Plasma TG levels and TG kinetics determined by the triton WR1339 method are shown in Table 2. The TG level was significantly higher in diabetic WT mice than in nondiabetic WT mice. Apo CIII–null mice had significantly lower plasma TG levels compared with WT mice. Unlike WT mice, diabetes did not increase the plasma TG level in apo CIII–null mice, and there was no difference of plasma TG levels between nondiabetic and diabetic apo CIII–null mice. Diabetes tended to decrease the TGSR in WT mice, but this change was not significant. Apo CIII–null mice had a 2-fold higher TGSR than WT mice. STZ-induced diabetes significantly decreased TGSR

Table 2. TG Kinetics Determined by the Triton WR1339 Method in WT and Apo CIII–Null Mice With and Without Diabetes

	WT		Apo CIII–Null	
	Nondiabetic	Diabetic	Nondiabetic	Diabetic
n	15	12	14	13
Plasma TG (mg/dL)	65 ± 33	102 ± 29*	35 ± 9*‡	35 ± 8*†
TGSR (mg/min)	0.010 ± 0.009	0.006 ± 0.002	0.020 ± 0.016*‡	0.011 ± 0.009†
FCR (pools/min)	0.016 ± 0.010	0.007 ± 0.004*	0.076 ± 0.105*	0.037 ± 0.038*†‡

*$P < .05$ *v* nondiabetic WT.
†$P < .05$ *v* nondiabetic apo CIII–null.
‡$P < .05$ *v* diabetic WT.

in apo CIII–null mice, so that it was comparable to the TGSR in nondiabetic WT mice.

FCR was estimated by calculation of the TGSR and TG pool size. FCR was significantly reduced in diabetic WT mice, falling to half of that in nondiabetic WT. Apo CIII–null mice had a 5-fold higher FCR than WT mice. STZ-diabetes also halved the FCR in apo CIII–null mice. However, the FCR of diabetic apo CIII–null mice was still 2.3-fold greater than that of nondiabetic WT mice.

VLDL Clearance

To determine if the nature of VLDL particles played a significant role in TG removal, we examined the plasma clearance of tritiated VLDL-TG obtained from WT mice or apo CIII–null mice with or without diabetes. The mean percentages of residual tritiated VLDL-TG obtained from different donor mice in WT or apo CIII–null recipient mice are depicted in Fig 1. The reinjection study demonstrated that VLDL-TGs derived from nondiabetic WT, diabetic WT, nondiabetic apo CIII–null, or diabetic apo CIII–null mice were cleared in a similar fashion from the plasma of nondiabetic WT recipients, and there was no difference of the residual percentage of injected VLDL at any sampling time among the groups. Similarly, VLDLs derived from nondiabetic WT, diabetic WT, nondiabetic apo CIII–null, or diabetic apo CIII–null mice were cleared in a similar fashion from the plasma of apo CIII–null recipients. Although we did not find any difference in the plasma clearance of VLDL-TG from different donors, all VLDL-TG obtained from the 4 different donors was cleared significantly faster by apo CIII–null recipients than by WT recipients. Thus, the percentage of residual tritiated VLDL-TG was always significantly lower in apo CIII–null recipients than in WT recipients, irrespective of the VLDL donor.

LPL Activity in Postheparin Plasma

Figure 2 shows LPL activity in plasma at 10 minutes after the injection of heparin. The activity tended to be lower in diabetic WT mice than in normal WT mice, but this difference did not attain significance. LPL activity was not increased in apo CIII–null mice, but was comparable to that in WT mice.

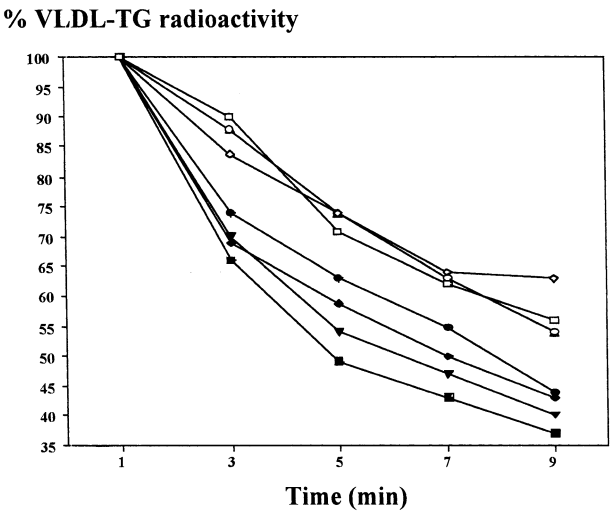

Fig 1. Mean percent of residual radioactivity of [^{3}H]VLDL-TG from various donors in WT and apo CIII–null recipient mice when the radioactivity at 1 minute was 100%. Open and closed symbols indicate the radioactivities in WT and apo CIII–null recipients, respectively. Squires, triangles, diamonds and circles indicates VLDL from nondiabetic WT, nondiabetic apo CIII–null, diabetic WT, and diabetic apo CIII–null donor mice, respectively.

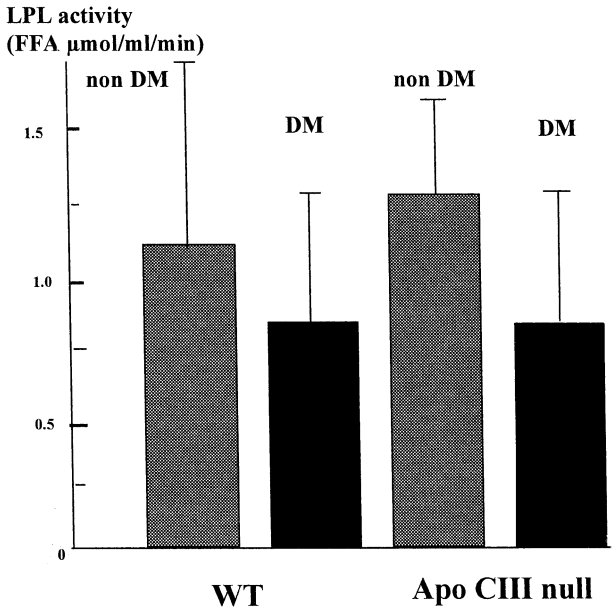

Fig 2. No significant differences of LPL activity in postheparin plasma in WT and apo CIII–null mice in the presence or absence of STZ-induced diabetes mellitus (DM). Data represent mean ± SD.

Diabetes slightly decreased the LPL activity in apo CIII–null mice, but this difference also did not attain significance.

DISCUSSION

We observed that the plasma TG level was significantly elevated but TGSR was not increased in normal mice (WT) when insulin-deficient diabetes was induced by the injection of STZ. Insulin plays an essential role in activating lipogenic enzymes in the liver, and conversely, hepatic TG production is markedly impaired in the insulin-deficient state. Sparks et al[27] reported that apo B, an essential apolipoprotein for VLDL assembly, is substantially degraded in the hepatocytes of STZ-induced diabetic rats, thereby inhibiting the assembly of VLDL. Most TG kinetic studies in STZ-induced diabetic rats have revealed that the plasma TG level is elevated, but the TGSR is not increased.[5-8] Therefore the hypertriglyceridemia in these diabetic animals is exclusively attributable to a defect of TG catabolism. We recently reported that apo CIII–null mice showed higher TG production than WT mice.[20] Since apo CIII inhibits uptake of TG-rich lipoprotein particles by the liver,[14,17] lack of apo CIII would facilitate TG flux into the liver, and would subsequently stimulate VLDL secretion to avoid excess hepatic accumulation of TG. Alternatively, apo E may be increased on the VLDL particles obtained from apo CIII–null mice by the replacement of soluble apolipoproteins,[11,12,14] which might result in enhancing apo E–mediated particle uptake and subsequently stimulating VLDL assembly and secretion. Jong et al[28] recently reported that there is no significant difference in TG production between apo CIII–null and WT mice. We cannot readily explain the difference between Jong et al[28] and our results. Diabetes reduced the higher TGSR in apo CIII–null mice to the level seen in normal WT mice. The normal TGSR value in diabetic apo CIII–null mice suggested that an increase of TG-rich lipoprotein flux into the liver could overcome decreased hepatic lipogenesis and increased apo B degradation in insulin-depleted animals.

Maeda et al[15] first established apo CIII–null mice and studied their TG metabolism. They reported that the plasma clearance of radiolabeled chylomicron-TG was significantly faster in these mice than in WT mice. They also demonstrated that the postprandial increase of TG after a fat load was substantially suppressed in apo CIII–null mice. On the other hand, several studies have demonstrated that human apo CIII transgenic mice develop severe hypertriglyceridemia due to a defect in TG removal.[11-13] Marcoux et al[29] reported that TG-rich lipoprotein–apo CIII levels are strongly correlated with TG-rich lipoprotein–TG levels in men. Batal et al[30] reported that overproduction of apo CIII plays a critical role in human with hypertyriglyceridemia. Taken together, it is highly possible that apo CIII is a critical regulator of the plasma clearance of TG-rich lipoproteins. It is particularly interesting that Ebara et al[13] have shown that apo CIII transgenic mice with disruption of the apo E gene have much higher plasma TG levels than apo E–null mice with a normal apo CIII gene. This study suggests that apo CIII can directly inhibit TG-rich lipoprotein catabolism, independently of apo E. Bar-on et al[3,4] demonstrated that VLDL-TG from STZ-induced diabetic rats was cleared more slowly than VLDL-TG from normal rats in normal recipient rats. They also demonstrated that apo CIII was increased, but apo E was decreased, in VLDL from STZ-diabetic rats,[2,4] suggesting that increased apo CIII/apo E ratio is due to a compositional abnormality of VLDL that is responsible for delayed catabolism. Chen et al[31] have demonstrated that hepatic apo CIII mRNA was increased in STZ-diabetic mice and that insulin treatment induced dose-dependent downregulation of apo CIII transcriptional activity, suggesting the existence of an insulin response element in the apo CIII gene. It has been proposed that apo CIII and apo E can replace each other in VLDL particles,[11,12,14] so it is possible that an abundance of apo CIII leads to a decrease of apo E in the VLDL particles of insulin-deficient diabetic animals. The present study demonstrated that the plasma TG level was unchanged in apo CIII–null mice after the development of diabetes, suggesting that apo CIII is an obligatory factor for the onset of hypertriglyceridemia in STZ-diabetic animals. Measurement of apoprotein composition in VLDL will be required to elucidate this possibility.

It is well known that the activity of LPL, a rate-limiting enzyme for TG hydrolysis, is decreased in diabetic patients.[32] Unlike humans, experimental studies have often failed to demonstrate a decrease of LPL in postheparin plasma from STZ-diabetic rats[6,9] or mice.[10] Similarly, we could not observe a significantly lower activity of postheparin plasma LPL in STZ-induced diabetic mice, although a defect of TG catabolism is present in these mice. Because a number of previous studies have demonstrated that LPL activity is decreased in uncontrolled or severe diabetes, this enzyme might be significantly decreased exclusively in diabetic mice having severe hyperglycemia. Apo CIII has been shown to inhibit the interaction of VLDL with heparan sulfate proteoglycans (HSPG) rather than being a direct inhibitor of LPL.[11-14] Therefore, it seems likely that an increase of apo CIII interferes with binding between VLDL and LPL anchored on HSPG in the vascular endothelium, resulting in reduced VLDL-TG hydrolysis. Ebara et al[21] have recently demonstrated that hepatic synthesis of HSPG was significantly suppressed in STZ-induced diabetic mice. Suppression of HSPG biosynthesis may enhance the inhibition of the VLDL-LPL interaction by apo CIII. We observed normal LPL activity in apo CIII–null mice, which is consistent with the normal LPL activity in apo CIII transgenic mice that was demonstrated elsewhere,[13] suggesting that apo CIII does not directly regulate LPL activity in vivo.

deSilva et al[12] reported that VLDL obtained from apo CIII transgenic mice is resistant to hydrolysis by bovine milk LPL in vitro. However, it remains to be determined whether this VLDL is cleared more slowly from the circulation. Ebara et al[13] demonstrated slow plasma clearance of VLDL obtained from human apo CIII transgenic mice. However, they used the same animals as recipients and VLDL donors for their VLDL turnover study, so it also remains unknown whether the slow clearance of VLDL from apo CIII transgenic mice is attributable to the nature of VLDL particles. In this study, we did not find rapid clearance of VLDL obtained from apo CIII–null mice. It is possible that rapid exchange of apolipoproteins between injected VLDL and the recipient's lipoproteins occurs during the turnover study, which may mask the abnormal nature of the VLDL particles. Thus, apolipoprotein exchange would be critical in our reinjection studies. However, it still

remains a possibility that abnormalities of VLDL may determine its plasma clearance if the exchange of apolipoprotein between lipoproteins is inactive.

The VLDL-TG clearance study demonstrated that all types of VLDL were cleared faster by apo CIII–null recipients than WT recipients, regardless of VLDL donor. Jong et al[28] have recently reported that TG removal was significantly faster in apo CIII–null mice when an artificial TG emulsion was used as a tracer, indicating that the TG removal system is activated in apo CIII deficiency, a finding which is in good agreement with our results. We did not examine VLDL-TG clearance using STZ-diabetic mice as the recipients in the present study. However, the low FCR determined by the triton method and the normal clearance of VLDL from the diabetic mice strongly suggests that the TG removal system is impaired in STZ-diabetic mice. Furthermore, we previously confirmed a slow removal rate of TG in STZ-diabetic rats using Intralipid or VLDL-TG from normal rats as tracers.[5,6,8] Accordingly, it seems likely that the TG catabolic defect in STZ-diabetic mice is more closely associated with the problem of removal system of TG-rich lipoproteins than with the abnormal nature of the lipoprotein particles. How does apo CIII affect TG removal system, besides causing a change of lipoprotein particle composition? We speculate that increased hepatic apo CIII production may directly impair the hepatic uptake of TG-rich lipoproteins by the liver through inhibiting the interaction of these lipoproteins with lipoprotein receptors or HSPG, but further studies are required to investigate our hypothesis. The present study suggests that apo CIII plays a critical role in the onset of hypertriglyceridemia in animals with insulin-deficient diabetes. Studies injecting VLDLs from nondiabetic and diabetic donors into diabetic recipients (both WT and apo CIII–null) will be required to further elucidate the role of VLDL particle and TG removal system in the development of hypertriglyceridemia associated with diabetes mellitus.

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