

Effect of dietary cholesterol and alloxan-diabetes on tissue cholesterol and apolipoprotein E mRNA levels in the rabbit

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Abstract Alloxan-diabetic rabbits develop hypercholesterolemia and hypertriglyceridemia in response to cholesterol feeding. To determine whether alterations in apolipoprotein composition of plasma lipoproteins were due to changes in apolipoprotein gene expression, we measured the steady state apoE mRNA levels in several tissues from both control and diabetic rabbits. Control rabbits were fed either chow or chow plus 1.5% cholesterol (chow-fed or cholesterol-fed groups) and diabetic rabbits were fed either chow or chow plus 0.5% cholesterol for dietary periods of 5, 21, and 42 days. The tissues examined were liver, small intestine, brain, adrenals, and aorta. ApoE mRNA levels were measured by Northern and dot blot analysis with a human apoE cDNA probe. In control rabbits fed either chow or cholesterol diets for up to 42 days, the steady state apoE mRNA levels remained relatively constant in all of the tissues examined. In contrast, in alloxan-diabetic rabbits fed a 0.5% cholesterol diet, apoE mRNA was reduced in liver, brain, and adrenals ($46 \pm 19\%$, $56 \pm 5\%$, and $39 \pm 18\%$ of chow-fed control, respectively), but showed little change in the aorta ($91 \pm 22\%$ of chow-fed control). Despite a similar increase in plasma cholesterol, the cholesterol content of the liver and adrenals of cholesterol-fed diabetic rabbits were 20% and 50%, respectively, of that of the liver and adrenals in cholesterol-fed control rabbits. The result that apoE mRNA levels and tissue cholesterol content are altered in the diabetic cholesterol-fed rabbit suggests that insulin deficiency in the rabbit may influence apoE gene expression and tissue cholesterol homeostasis. — Lenich, C. M., A. V. Chobanian, P. Brecher, and V. I. Zannis. Effect of dietary cholesterol and alloxan-diabetes on tissue cholesterol and apolipoprotein E mRNA levels in the rabbit. *J. Lipid Res.* 1991. 32: 431–438.

Supplementary key words apolipoprotein metabolism

Several studies (1–5) have shown that while both normal and diabetic rabbits develop marked hypercholesterolemia in response to cholesterol feeding, the severity of atherosclerosis in cholesterol-fed diabetic rabbits is markedly reduced compared to nondiabetic animals. Comparative studies of the plasma lipoproteins associated with the hypercholesterolemic diabetic and nondiabetic rabbits demonstrated major chemical and metabolic

differences (6, 7). Diet-induced hypercholesterolemia in nondiabetic rabbits results in the accumulation of abnormal, cholesteryl ester-rich and apoprotein E-rich very low density lipoproteins (8–10) that are readily metabolized by cultured macrophages (11). In contrast, the plasma lipoproteins that accumulate in cholesterol-fed alloxan-diabetic rabbits are triglyceride-rich and contain less apoE and more of the apolipoproteins A-I, A-IV, and B-48 than those of nondiabetic cholesterol-fed rabbits (12). When incubated with mouse peritoneal macrophages, these lipoproteins from diabetic animals did not stimulate the formation of cellular cholesteryl esters (7).

The mechanism by which dietary cholesterol induces hypercholesterolemia has been only partially characterized in either the normal or diabetic rabbit model. Despite the 17-fold increase in plasma apoE found in normal cholesterol-fed rabbits, the rate of hepatic apoE synthesis is not markedly increased (13). Additionally, when hepatic apoprotein mRNA levels were measured in normal chow- and cholesterol-fed rabbits, no change was detected in the level of apoE mRNA (14–16), and apoB mRNA levels were lower in the cholesterol-fed rabbits, indicating a reduced ability to synthesize apoB and VLDL (17). Kovanen et al. (18) have demonstrated that hepatic lipoprotein receptors are both saturated and down-regulated in the normal cholesterol-fed rabbit, suggesting that diet-induced hypercholesterolemia is mainly due to a decreased receptor-mediated catabolism of cholesterol-rich apoE-containing lipoprotein particles. While a reduced clearance of plasma triglyceride has been demonstrated in diabetic rats (19), the nature of this defect is not clear. Neither apolipoprotein synthetic rates nor apolipoprotein mRNA levels have been measured in tissues from the diabetic rabbit.

Abbreviation: VLDL, very low density lipoprotein.

We recently described the abundance, size, and distribution of several apolipoprotein mRNAs in the chow-fed rabbit using human apolipoprotein cDNA clones (20). In agreement with others (21, 22), apoE mRNA of approximately 1.2 kb was found to be present in a variety of tissues with liver being the major site of synthesis. ApoE functions as a ligand in mediating the uptake of chylomicron remnants and apoE-containing lipoproteins by hepatic and extrahepatic tissues (23–25). In this study we have determined apoE mRNA levels in the liver and in several other apoE-producing tissues in response to alloxan-diabetes and to cholesterol feeding. We have observed lower steady state apoE mRNA levels in the diabetic cholesterol-fed rabbit compared to those in the normal cholesterol-fed rabbit which can account for the reported deficiency of plasma apoE in this animal model.

MATERIALS AND METHODS

Materials

Radiolabeled nucleotides [^{32}P]dATP, [^{32}P]dCTP, and [^{32}P]dTTP were purchased from New England Nuclear. The Klenow fragment of DNA polymerase I and the M13 sequencing primer were purchased from New England Biolabs. Agarose and Bio-Gel P-60 were obtained from Bio-Rad. Nitrocellulose filters were obtained from Schleicher & Schuell, Inc.

Animals and dietary treatments

Male New Zealand white rabbits (2–2.5 kg) were purchased from Millbrook Farms (Amherst, MA). The animals were housed individually and fed Purina Rabbit Chow (Ralston Purina Co., St. Louis, MO) ad lib for 1 week before any treatment. To produce diabetes, animals were administered alloxan monohydrate (150 mg/kg) by infusion of a 0.25 M aqueous solution through the lateral ear vein at a rate of 1 ml per min. Twenty-four hours after alloxan treatment, rabbits with plasma glucose levels 250 mg/ml were fed either chow alone or chow containing 0.5% cholesterol and 6% corn oil (chol diet) for 5, 21, or 42 days. Control animals were not given alloxan but were fed either chow alone or chow containing 1.5% cholesterol and 6% corn oil (chol diet) for the same time periods. Fasting blood samples were taken from the lateral ear vein and collected in 0.1% EDTA for measurement of glucose and lipid levels. At the end of the treatment period, animals were fasted overnight and killed by injection of an overdose of Nembutal. The effect of an acute triglyceride bolus on hepatic and intestinal apolipoprotein mRNA levels was also examined in nondiabetic rabbits. In these studies, rabbits were killed by Nembutal injection after either an overnight fast (16 h) or 3 h after being fed chow containing 6% corn oil. In both studies, tissues were quickly removed from the animals and stored at -70°C or immediately prepared for RNA isolation.

RNA isolation and RNA blot hybridization

Total cellular RNA was isolated from rabbit tissues as described previously (22). Briefly, homogenized tissues were centrifuged in a solution of guanidinium thiocyanate and cesium chloride to extract and pellet total RNA. The RNA pellet was then washed by ethanol precipitation, dissolved in double-distilled water, and stored at -70°C . The integrity of each preparation of RNA was assessed visually after electrophoresis through agarose gels containing ethidium bromide (0.5 $\mu\text{g}/\text{ml}$). The RNA was then transferred onto nitrocellulose filters, baked, and hybridized to ^{32}P -labeled apolipoprotein cDNA probes. Hybridization was done at 42°C for 16–24 h in a solution containing 50% deionized formamide, 0.75 M sodium chloride, 0.15 M Tris base, 2 mM sodium pyrophosphate, 0.15 M sodium phosphate, 0.1% SDS, $10\times$ Denhart's solution (2% Ficoll, 2% polyvinylpyrrolidone, 2% bovine serum albumin), 20 $\mu\text{g}/\text{ml}$ sheared single-stranded salmon sperm DNA, and 10% dextran sulfate. Filters were washed in 0.1 M SSC, 0.1% SDS twice at room temperature (10 min each) and finally at 55°C for 30 min. Dot blot analysis of RNA was done as previously described (26). All RNA samples from diabetic and control rabbits were included in the same blot. Three separate blots were prepared. The relative concentration of apolipoprotein mRNA was estimated by scanning densitometry of the autoradiograms. The exposure time was selected to ensure linear relationship of the absorbance versus concentration.

Preparation of apolipoprotein DNA probes

Apolipoprotein DNA probes were prepared using single-stranded M13 clones as templates as described by Zannis et al. (22). Briefly, 0.2–1 μg of the appropriate single-stranded M13 apolipoprotein DNA template was mixed with 2.5 pmol of sequencing primer and 0.5 μl of a buffer containing 30 mM Tris-HCl, pH 7.5, 50 mM NaCl, 15 mM MgCl_2 , and 1 mM dithiothreitol. The mixture was heated at 65°C for 30 min and left at room temperature for 60 min. The above mixture was transferred to a tube containing 50 pmol each of ^{32}P -labeled dATP, dCTP, and dTTP, 50 pmol of unlabeled dGTP, and 5 μg nuclease-free bovine serum albumin. Klenow fragment of DNA polymerase I (0.5 unit) was added and the mixture was then incubated for 30 min on ice and an additional 60 min at room temperature. The radiolabeled probe was purified by phenol extraction and Bio-Gel P-60 chromatography. The single-stranded DNA templates used were as follows: *a*) a clone containing the PstI to PstI fragment of the human apoA-I gene (27) inserted into the PstI site of M13 mp11 vector; *b*) a clone containing the cDNA sequence of a gtA-IV clone (28) inserted into the EcoRI site of M13 mp11 vector; and *c*) a clone containing the first 5' upstream 0.45 kilobase PstI-PstI fragment of the apoE cDNA clone pE-368 (29) inserted in the PstI to PstI site of the M13 mp11 vector.

Lipid analysis

Total plasma triglyceride and cholesterol concentrations were determined enzymatically (Sigma Triglyceride Kit No. 336 and Sigma Cholesterol Kit No. 352, St. Louis, MO). Tissues were extracted for lipid analysis according to the method of Folch, Lees, and Sloane Stanley (30). Tissue cholesterol was determined by the method of Rudel and Morris (31). Plasma glucose was measured using a Glucose Analyzer (Perkin-Elmer, double-beam spectrophotometer, Coleman 124).

RESULTS

Expression of hepatic and intestinal apolipoprotein mRNAs in response to acute triglyceride feeding

To examine the effect of dietary triglyceride on mRNA, RNA was isolated from chow-fed rabbits after either an overnight (16 h) fast or 3 h after ad lib ingestion of a chow meal supplemented with corn oil (6%). The relative amounts of apoA-I, apoA-IV, and apoE mRNAs in liver and small intestine were examined by Northern blot analysis using human apolipoprotein cDNA probes (**Fig. 1**). This analysis showed that intestinal and hepatic apoA-IV mRNA increased significantly in response to triglyceride feeding (two- to sevenfold) whereas there was smaller increase in intestinal apoA-I mRNA and no increase in hepatic apoE mRNA.

Effect of cholesterol-feeding on plasma and tissue cholesterol levels in normal and diabetic rabbits

Animals to be made diabetic were given alloxan and, along with untreated control animals, were placed on a cholesterol diet for 5, 21, or 42 days. To induce comparable plasma cholesterol levels, diabetic rabbits were

TABLE 1. Plasma lipid and glucose levels in control and diabetic rabbits fed chow or a cholesterol-containing diet

Animals and Diet	No.	Duration	Plasma Level		
			Chol	TG	Glucose
		<i>days</i>		<i>mg/dl</i>	
Nondiabetic					
Chow	2	5	41	58	100
Chow	2	21	46	46	80
Chow	2	42	26	38	ND
Chol	3	5	671	65	90
Chol	3	21	1,323	236	110
Chol	2	42	1,519	237	ND
Diabetic					
Chow	2	5	278	360	368
Chow	2	21	236	230	380
Chol	3	5	775	3,181	320
Chol	2	21	896	4,678	400
Chol	1	42	1,820	6,488	400

The cholesterol content of the diet was 1.5% for the nondiabetic and 0.5% for the alloxan-treated rabbits. Measurements were performed on samples obtained from fasted animals on the day they were killed. Values were expressed as group means. Chol, cholesterol; TG, triglyceride; ND, not determined. The ranges of cholesterol values in mg/dl were: a) nondiabetic: on chow diets, 21-63; on 5-day cholesterol diet, 500-774; on 21- and 42-day cholesterol diet, 1028-1858; b) diabetic: on chow diet, 108-439; on 5-day cholesterol diet, 576-990; on 21- and 42-day cholesterol diet, 650-1820.

fed chow containing 0.5% cholesterol while control animals received chow containing 1.5% cholesterol (7). The differences of dietary cholesterol were necessary to produce similar levels of plasma cholesterol in the two study groups. Of the 34 animals that were alloxan treated, only 10 diabetics survived the dietary treatment periods and were distributed in groups as shown in **Table 1**. As expected, the plasma cholesterol levels of both cholesterol-fed diabetic and nondiabetic animals increased markedly and to a similar extent throughout the 42-day treatment period. In addition, the plasma cholesterol level of chow-fed diabetic rabbits was greater than that of nondiabetic chow-fed rabbits, but did not increase as dramatically as that of the cholesterol-fed control or diabetic rabbits. Plasma triglyceride levels also increased markedly in the cholesterol-fed diabetic rabbits and to a lesser extent in the chow-fed diabetics compared to chow- and cholesterol-fed nondiabetics, respectively.

After being maintained on either the chow or cholesterol diet for 5, 21, or 42 days, animals were killed and tissues from each animal were removed for cholesterol analysis and mRNA isolation. As shown in **Fig. 2**, a large increase in the cholesterol content of the liver and adrenal glands of the cholesterol-fed control rabbits was apparent. In contrast, despite similar plasma cholesterol levels, the levels of cholesterol in the cholesterol-fed diabetic rabbits were lower in both the liver and the adrenal glands as compared to the cholesterol-fed nondiabetic animals.

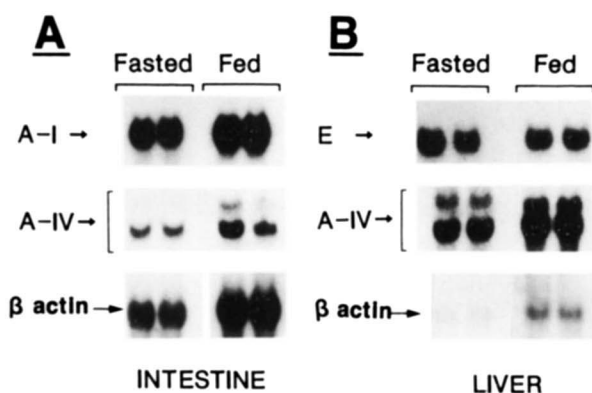


Fig. 1. Blotting analysis of RNA isolated from rabbit liver and intestine. Panels A and B represent analysis of RNA isolated from small intestine and liver, respectively, of chow-fed or fasted rabbits. Rabbits were killed after an overnight fast (16 h) or 3 h after ingestion of chow containing 6% corn oil. Northern blots were hybridized with ³²P-labeled human apoA-I, apoA-IV, apoE, and chicken beta-actin cDNA probes, as described in Methods, washed, and exposed to X-ray film at -70°C. Each lane contained 30 μg of RNA pooled from three animals.

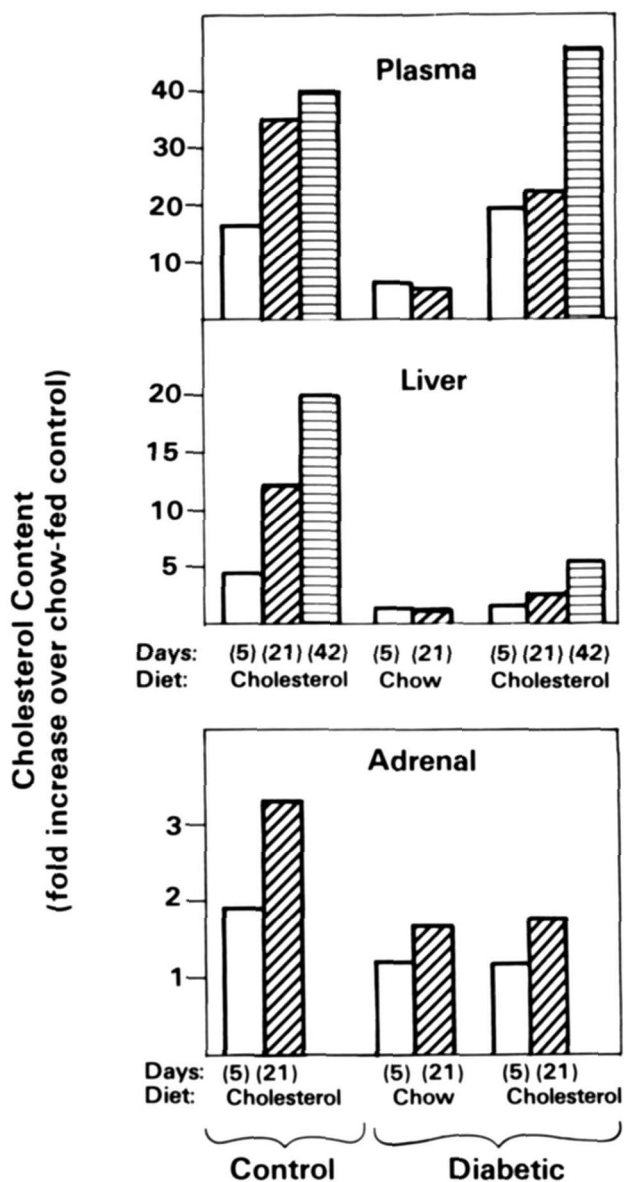


Fig. 2. Cholesterol content of the plasma, liver, and adrenal gland of control (CON) and diabetic (DIAB) rabbits fed chow or a cholesterol (chol) diet for 5, 21, or 42 days as described in Methods. Data are expressed as the fold increase over chow-fed control values and are averages of two or three animals. Adrenal cholesterol content for the 42-day treatment group was not determined. The mean cholesterol values of the chow-fed control rabbits were: plasma, 38 mg/dl; liver, 2.4 mg/g wet wt; and adrenal, 95 mg/g wet wt.

Statistical analysis of pooled data of days 5 and 21 showed that the liver cholesterol content of the cholesterol-fed control group was fivefold higher than that of the cholesterol-fed diabetic animals (21 ± 12 vs. 4 ± 1 mg/g wet wt, mean \pm SD, $P < 0.02$). Analyses of the pooled data (5 and 21 days) of adrenal cholesterol content showed that the adrenal cholesterol content of the cholesterol-fed control was 1.8-fold higher than that of the cholesterol-fed diabetic animals (248 ± 108 vs. 141 ± 33 mg/g wet wt,

mean \pm SD) but that this difference was not statistically significant.

Effect of cholesterol-feeding on the expression of apoE mRNA in tissues of normal and diabetic rabbits

The effects of cholesterol feeding and alloxan-diabetes on apoE mRNA levels were determined by Northern and dot blot analysis. RNA from the liver, small intestine, brain, adrenal glands, and aorta of control and diabetic animals was used to prepare a series of Northern blots which were hybridized with a 32 P-labeled apoE cDNA probe (29). Representative autoradiograms of the Northern blots are shown in Figs. 3 and 4. This qualitative analysis indicated that apoE mRNA levels did not change in any of the tissues examined from nondiabetic rabbits throughout the 42 days of cholesterol-feeding (Fig. 3). In contrast, within 5 days of alloxan treatment, apoE mRNA levels were reduced in the liver, brain, and adrenal glands of both the chow and cholesterol-fed diabetic rabbits (Fig. 4). RNA blots from each tissue were quantified by scanning densitometry and the results are summarized in Table 2. This analysis indicated that apoE mRNA levels in both the chow and cholesterol-fed diabetic rabbits were significantly lower than the apoE mRNA levels in the chow or cholesterol-fed nondiabetic rabbits. The greater reduction in apoE mRNA levels was observed in the cholesterol-fed diabetic animals where apoE mRNA in the liver, brain, adrenal glands, respectively, was $46 \pm 19\%$, $54 \pm 5\%$, $39 \pm 18\%$ of that found in the corresponding tissues of the chow-fed nondiabetic controls. In contrast to these three tissues, apoE mRNA levels in the aorta and small intestine (data not shown) of both chow and cholesterol-fed diabetics remained at a low and relatively constant level.

In control experiments, liver apoE mRNA was quantified by dot blots using several concentrations of representative hepatic RNA samples. Consistent with the

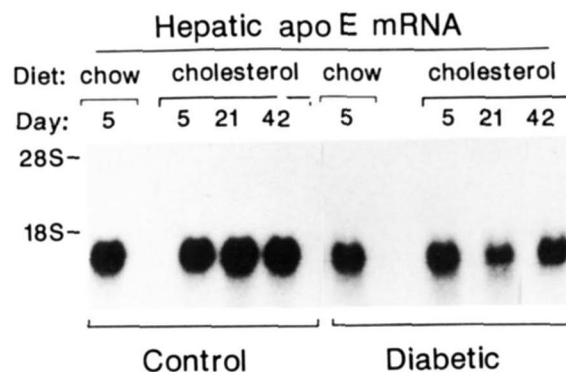


Fig. 3. Blotting analysis of hepatic RNA obtained from normal and diabetic rabbits fed either chow or a cholesterol-containing diet for the number of days indicated. Each lane contained 20 μ g of total RNA. The positions of 28S and 18S ribosomal RNAs are indicated.

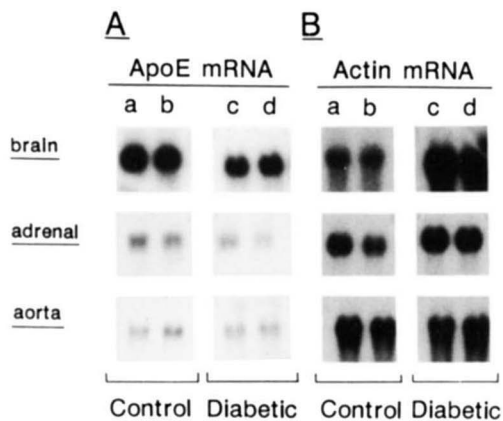


Fig. 4. Blotting analysis of rabbit apoE RNA isolated from rabbit brain, adrenal glands, and aorta of control (lanes a and b) and diabetic (lanes c and d) rabbits fed a cholesterol-containing diet for 5 days. Panel A shows representative blots hybridized with ^{32}P -labeled apoE cDNA, as described in Methods. Panel B shows the same lanes following hybridization with a ^{32}P -labeled rat beta-actin cDNA probe.

results of Table 2, which are based on Northern blotting, this analysis showed that apoE mRNA in both the chow and cholesterol-fed animals was $42 \pm 9\%$ and $28 \pm 18\%$ of that found in the chow or cholesterol-fed nondiabetic rabbits, respectively.

Representative Northern and dot blots discussed above were reprobbed with a rat beta-actin cDNA probe (Figs. 4A and B). This analysis showed that, in spite of the reduction of apoE mRNA levels, the beta-actin mRNA of diabetic rabbits either did not change (aorta) or was increased (brain and adrenals).

DISCUSSION

The results presented here demonstrated that steady state apoE mRNA levels were reduced in the liver, brain, and adrenal glands of chow- and cholesterol-fed diabetic

rabbits compared to controls. The change occurred within 5 days of alloxan treatment and coincided with the onset of hyperlipidemia in these animals.

ApoE mRNA levels did not change in the liver, intestine, brain, adrenal glands, or aorta of nondiabetic rabbits in response to dietary cholesterol. Additionally, hepatic and intestinal apoE mRNA levels were not affected by an acute triglyceride bolus which, in contrast, did increase apoA-IV and beta-actin mRNA in these tissues of the nondiabetic rabbit (Fig. 1). The regulation of intestinal apoA-IV mRNA by dietary triglyceride has previously been demonstrated in the rat (32, 33). Elshourbagy et al. (34) recently reported that apoA-IV mRNA levels in both small intestine and liver of neonatal rats correlate with their triglyceride secretion rates, suggesting a role for both hepatic and intestinal apoA-IV in the metabolism of dietary triglyceride.

In the present study apoE mRNA did not increase in either the liver or adrenal glands of nondiabetic rabbits in response to dietary cholesterol despite a 19- and 3-fold increase in the cholesterol content of these tissues, respectively. Others have demonstrated that dietary cholesterol has no effect on hepatic apoE mRNA in the rabbit (16), mouse (35, 36), and rat (37). Cholesterol content has been shown to regulate apoE metabolism in mouse peritoneal macrophages where a small increase in cellular free cholesterol stimulated apoE synthesis and mRNA levels in a dose-dependent manner (38).

An interesting aspect of the present study is the difference in the cholesterol content of tissues of normal and diabetic rabbits in response to cholesterol feeding. A similar reduction in cholesterol content was previously observed in the aortas of cholesterol-fed diabetic rabbits (1, 7). The levels of cholesterol in tissues of the cholesterol-fed alloxan-diabetic rabbits were lower five- and two-fold, respectively, than those of the cholesterol-fed control rabbits. These results are consistent with our previous observation that lipoproteins from diabetic rabbits failed

TABLE 2. Effect of alloxan treatment and cholesterol feeding on apoE mRNA levels in the rabbit

Animals and Diet	No.	Relative ApoE mRNA Levels			
		Liver	Brain	Adrenals	Aorta
Nondiabetic					
Chow	6	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.17	1.00 ± 0.12
Chol	8	1.01 ± 0.20	0.99 ± 0.13	1.10 ± 0.15	1.00 ± 0.26
Diabetic					
Chow	4	0.70 ± 0.14^a	0.71 ± 0.17^a	0.49 ± 0.18^a	ND
Chol	6	0.46 ± 0.19^b	0.54 ± 0.05^c	0.39 ± 0.18^b	0.91 ± 0.22

Alloxan-diabetic and control rabbits were fed chow or a cholesterol-containing diet as described in the legend to Fig. 2. Data represent relative levels of apoE mRNA estimated by densitometric scanning as described in Methods. Values are means \pm SD of pooled data of days 5, 21, and 42. Chol, cholesterol; ND, not determined. In the tissues examined, there were no statistical differences in apoE levels between chow- and cholesterol-fed groups for either nondiabetic or diabetic rabbits.

^a*P*, 0.025; ^b*P*, 0.01; and ^c*P*, 0.001 versus corresponding value for chow-fed nondiabetic controls.

to stimulate cholesterol esterification in cultured macrophages (7) and suggest that lipoprotein uptake was impaired. A defective clearance of S_f 400 and VLDL lipoproteins from plasma has been demonstrated in other animal models for experimental diabetes (19, 39) and in humans with insulin-dependent diabetes (40).

Several factors probably contribute to the lipoprotein abnormalities associated with diabetes. A reduction in plasma apoE could result in the defective clearance of triglyceride-rich lipoproteins since this protein is required for the receptor-mediated uptake of intestinal lipoproteins by liver (23, 24). It has been reported that individuals with a genetic apoE deficiency display a hyperlipemia resulting from the delayed clearance of triglyceride-rich intestinal and hepatic lipoproteins (41). In addition, lipoprotein lipase activity, which hydrolyzes triglyceride of intestinal and hepatic lipoproteins prior to uptake by liver, is reduced in diabetics (42, 43). A reduction has been observed in plasma apoC-II levels in diabetic rats (43). Finally, a reduced level of plasma membrane receptors, which mediate the cellular uptake of lipoproteins, could also contribute to the defect. While lipoprotein receptors have been shown to be reduced in the cholesterol-fed normal rabbit (18), their level has not been examined in the diabetic animal.

The function of apoE synthesized by the brain, adrenal glands, and other extrahepatic tissues is not clearly understood. However, several studies have shown that HDL is involved in cholesterol efflux from cells (44, 45). Association of the newly secreted apoE with HDL can lead to formation of HDL with apoE (46, 47), which can be catabolized by both hepatic and extrahepatic tissues (48, 49). This process has been characterized as reverse cholesterol transport (50). The net effect of formation and catabolism of apoE-containing lipoprotein particles could be the redistribution of dietary and newly synthesized cholesterol to the various cells and tissues and maintenance of cholesterol homeostasis.

A role for insulin in regulating apolipoprotein metabolism has been demonstrated in other studies using the alloxan-diabetic rabbit where abnormalities in the metabolism of triglyceride-rich lipoproteins (12) and HDL (51) were reversed by insulin treatment. In a recent study, insulin treatment had no effect on the steady state apoE mRNA levels of primary cultures of rat hepatocytes (34). The molecular mechanisms responsible for the reduction of apoE mRNA levels in the tissues of diabetic rabbit require further investigation. ■

This work was supported by NIH grant HL-18318 and by a grant from the Juvenile Diabetes Foundation #1881195. Vassilis I. Zannis is an Established Investigator of the American Heart Association. We are grateful to Dr. Steve Farmer for providing the rat beta-actin cDNA probe.

Manuscript received 28 June 1990 and in revised form 13 November 1990.

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