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Increased proportion of plasma apoB-48 to apoB-100 in non-insulin-dependent diabetic rats: contribution of enhanced apoB mRNA editing in the liver

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Abstract To assess the alteration of apolipoprotein (apo) B mRNA editing in non-insulin-dependent diabetes mellitus (NIDDM), we measured plasma apoB-100 and apoB-48 levels and apoB mRNA editing efficiency in the liver and intestine from GK (Goto-Kakizaki) rats, a genetically NIDDM animal. Male GK rats and control littermates, aged 25 weeks, were used in this study. Ventromedial hypothalamus (VMH)-lesioned control rats were used as hyperinsulinemic models. VMH-lesioned GK rats (GK + VMH) were treated as an insulin-exhausted NIDDM model. Plasma cholesterol and triglyceride levels were increased in GK rats. Very low density lipoprotein (VLDL)-triglyceride and low density lipoprotein (LDL)-cholesterol concentrations were significantly higher in GK rats than in controls. The increase of VLDL-triglyceride was most marked in GK + VMH rats. Plasma apoB-48 levels, quantified by immunoblot, were increased in GK rats. However, apoB-100 levels were minimally elevated in GK rats. Therefore, the apoB-48/apoB-100 ratio was remarkably increased in GK rats. ApoB mRNA editing was analyzed by reverse transcriptase–polymerase chain reaction coupled with dideoxynucleotide chain termination assay. The ratio of apoB-48-type cDNA to apoB-100-type cDNA was significantly increased in the liver from GK rats compared with controls. Although the development of the VMH lesion increased plasma apoB-48 levels, it had no effect on the proportion of apoB-48-type to apoB-100-type cDNA in the liver from both GK and control littermates. ApoB mRNA in the intestine was almost totally edited (~95%). Intestinal apoB-48/apoB-100 cDNA ratio showed no significant difference among the four groups. ■ In conclusion, an enhanced apoB mRNA editing was indicated in the non-insulin-dependent diabetic rats, which might contribute to the increase of plasma apoB-48 levels.—Yamane, M., S. Jiao (N. Sho), S. Kihara, I. Shimomura, K. Yanagi, K. Tokunaga, S. Kawata, H. Odaka, H. Ikeda, S. Yamashita, K. Kameda-Takemura, and Y. Matsuzawa. Increased proportion of plasma apoB-48 to apoB-100 in non-insulin-dependent diabetic rats: contribution of enhanced apoB mRNA editing in the liver. *J. Lipid Res.* 1995. **36**: 1676–1685.

Supplementary key words apolipoprotein B • animal model • low density lipoproteins • insulin resistance

Numerous studies have reported that dyslipoproteinemia is associated with both insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) (reviewed in ref. 1). A high prevalence of coronary artery disease in diabetes might be accounted for in part by the coexisting dyslipoproteinemia. Especially, increased plasma very low density lipoprotein (VLDL)-triglyceride and decreased high density lipoprotein (HDL)-cholesterol are typical features of uncontrolled patients with IDDM or NIDDM. However, the precise mechanism of diabetic dyslipoproteinemia is still unknown. In previous studies, we indicated an enhancement of intestinal acyl-CoA:cholesterol acyltransferase activities in two types of diabetic animals, streptozotocin-induced diabetic rats with insulinopenia (2) and Wistar fatty rats with insulin resistance (3). As

Abbreviations: apo, apolipoprotein; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; VMH, ventromedial hypothalamus; IRI, immunoreactive insulin; FT₃, free triiodothyronine; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase–polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; bp, base pair; nt, nucleotides.

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this enzyme is thought to be an essential factor controlling the absorption of dietary cholesterol (4), we speculate that cholesterol absorption is enhanced in diabetes, resulting in a marked accumulation of chylomicron remnants in the circulation, especially under the condition of cholesterol feeding.

Apolipoprotein (apo) B is required for the intracellular assembly and secretion of exogenous and endogenous triglyceride-rich lipoproteins in both liver and intestine (5). There are two translation products of the apoB gene, designated as apoB-100 and apoB-48, in many mammals including rats (6, 7), mice (8), rabbits (9), and humans (10). Human apoB-100 (4536 amino acids) is synthesized predominantly by hepatocytes and integrated into VLDL and low density lipoprotein (LDL) as their constitutional apolipoprotein (11–13). Human apoB-48 (2152 amino acids), an amino terminal half part of apoB-100, is synthesized by intestinal epithelial cells and secreted into the lymph as a component of chylomicrons. Both peptides are coded by a single copy of the apoB gene. ApoB-48 is synthesized through the newly found mechanism, mRNA editing. A substitution of uracil for cytosine is post-transcriptionally introduced at the nucleotide position 6666 of apoB-100 mRNA, resulting in a change of the codon CAA encoding Gln²¹⁵³ to the stop codon UAA (14–16). Consequently, the translation of this apoB mRNA produces the shorter apoB-48.

LDL containing apoB-100, which can be recognized by the LDL (apoB,E) receptors, undergoes the receptor-mediated uptake at a relatively slow clearance rate (half life ~2.5 days). In contrast, apoB-48 cannot be recognized by the LDL receptors. ApoB-48-containing lipoproteins are catabolized by lipoprotein lipase and then cleared by cellular uptake through putative remnant receptors and apoE interaction in the liver, resulting in a rapid clearance from plasma (half life ~1–2 h) (reviewed in ref. 17). Thus, the subpopulations of apoB play a crucial role in the metabolic destinations of apoB-containing lipoproteins. In contrast to humans, a part of apoB-100 mRNA is edited to apoB-48-type mRNA in rodent hepatocytes, and consequently both apoB-100 and apoB-48 are synthesized by the liver and secreted into the circulation (18). Alteration in the efficiency of hepatic apoB mRNA editing can lead to changes of plasma lipoprotein profile. Actually, triiodothyronine treatment to rats causes the enhancement of hepatic apoB mRNA editing and increase of apoB-48 secretion, which leads to elevated plasma levels of apoB-48 (19). Furthermore, Leighton et al. (20) indicated that fasting suppresses apoB mRNA editing in rat hepatocytes and secretion of apoB-48. Dietary or metabolic factors might regulate apoB mRNA editing. However,

there have been no reports concerning apoB mRNA editing in diabetes mellitus.

In 1976, Goto, Kakizaki, and Masaki (21) established a new genetically diabetic animal designated as GK (Goto-Kakizaki) rats by selective inbreeding of Wistar rats using glucose intolerance as a selection marker. This animal model has characteristics of NIDDM such as hyperglycemia, insulin resistance, hyperinsulinemia, and diabetic complications such as nephropathy and neuropathy (22–24). Insulin resistance was demonstrated by oral or intravenous glucose challenge (24). The life span of GK rats is over 12 months, while BB rats, a spontaneous IDDM animal, die within 2 months after birth due to ketoacidosis, unless insulin is supplemented.

In the present study, to elucidate the effect of diabetes on apoB mRNA editing, we measured plasma apoB-48 levels and the efficiencies of hepatic and intestinal apoB mRNA editing in GK rats and compared the results to those of ventromedial hypothalamus (VMH)-lesioned obese rats as a hyperinsulinemic model. Furthermore, we recently observed that VMH-lesioned GK rats showed decreased pancreatic insulin content and suggested that the increased requirement for insulin secretion may lead to pancreatic islet β -cell exhaustion instead of hyperinsulinemia, which is similar to the state of patients with advanced NIDDM (25). The effect of VMH lesion on apoB mRNA editing was also studied.

MATERIALS AND METHODS

Animals

Eleven male GK rats were used for the study. As female GK rats do not develop glucose intolerance, they were eliminated from the current study. Eleven male littermates with normal plasma glucose concentration were adopted as control. GK rats were originally derived from the strain established in Tohoku University by Drs. Goto and Kakizaki (21) and maintained at Takeda Chemical Industries (Osaka) under specific pyrogen-free status. The animals were weaned at 4 weeks of age. They were kept on a laboratory chow diet (Clea rat chow CE-2, 352 kcal/100 g, Clea Japan Inc., Tokyo) containing 4.6 weight percent fat (polyunsaturated/saturated fatty acids ratio 1.4), 50% carbohydrate (mainly corn starch), 25.2% protein, and 0.089% cholesterol, and drank tap water ad libitum in a room maintained at 22 ± 2°C and illuminated from 8:00 AM to 8:00 PM. At 9 weeks of age, GK rats and control littermates were divided randomly into two subgroups, VMH-lesioned and sham-operated (five animals for sham-operation and six for VMH). The VMH-lesions were made using a double coordinate sys-

tem developed by Tokunaga et al. (26). In brief, coordinates for placing the electrode were determined with reference to the interaural line and the bregma. The top of the upper incisor bar was 3.5 mm above the interaural line. A stainless steel electrode (RNEBX 300 × 50 mm, David Kopf Instruments, CA) was placed according to the coordinates table previously described (27). A direct current of 2 mA for 30 sec was then passed cathodically. Sham operation was carried out without current. Food consumption and body weight were monitored daily. After operation they were maintained in the same condition as above for 16 weeks. Then, the animals were exsanguinated under anesthesia by intraperitoneal injection of pentobarbital sodium (50 mg/kg) after an overnight fast. Blood was collected in a tube containing 1.5 mg/ml of disodium EDTA and promptly centrifuged at 3000 rpm at 4°C for 15 min. Plasma obtained was mixed with 100 KIU/ml of aprotinin (Sigma, St. Louis, MO), 50 µg/ml of gentamycin sulfate, and 0.05% NaN₃. The liver specimen was removed to prepare RNA. After rinsing the lumen of small intestine with ice-cold phosphate-buffered saline, intestinal mucosa was scraped by a glass plate and frozen in liquid nitrogen.

Lipids and lipoprotein analysis

Total cholesterol and triglyceride were measured enzymatically using commercially available kits, Determiner TC5 and TG-S 555, respectively (Kyowa Medex Co. Ltd., Tokyo) (3). TG-S 555 system is not influenced by plasma glycerol, because glycerol is degraded prior to lipoprotein lipase reaction through the combined reactions of glycerol kinase, glycerol-3-phosphate oxidase, and peroxidase. VLDL (d < 1.006 g/ml) was isolated from plasma by preparative ultracentrifugation. HDL-cholesterol concentrations were measured by heparin-Ca²⁺ precipitation method. LDL-cholesterol was estimated by subtracting VLDL-cholesterol + HDL-cholesterol from plasma total cholesterol.

Chemical measurements

Plasma glucose and immunoreactive insulin (IRI) levels were measured by the previously described methods (2). Plasma free triiodothyronine (FT₃) concentrations were determined by radioimmunoassay.

Western blot of rat apoB

Aliquots of whole plasma (20 µl) were delipidated by ethanol-diethylether 3:1 (v/v) and diethylether at -20°C. Delipidated proteins were dissolved by boiling for 10 min in 200 µl 5% sodium dodecyl sulfate (SDS) in 20 mM ethylmorphorine-HCl, pH 8.5, 10 µl 2-mercaptoethanol, and 20 µl 1.5% bromophenol blue in glycerol, and one fifth volume of sample was subjected to 3-6% gradient SDS-polyacrylamide gel electrophoresis (28).

Separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, 0.45 µm pore size, cat no. 162-0114, Richmond, CA) by electroblotting at 35 V for 18 h. Nitrocellulose membranes were blocked by 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.2, incubated with affinity-purified rabbit anti-rat apoB IgG kindly provided by Drs. E. S. Krul and G. Schonfeld, Washington University School of Medicine, St. Louis, MO, and then incubated with 1:1000 diluted peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). apoB bands were colorized by incubation with 0.1% 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Bethesda Research Laboratory, Gaithersburg, MD) and 0.2% hydrogen peroxide and quantified by a reflection densitometer at wave length of 450 nm (Shimadzu CS-9000, Kyoto). Signal intensities of apoBs were linear in the range of 1-150 µl of control rat plasma.

Oligonucleotides

The following nucleotides were synthesized on a Applied Biosystems 380B DNA synthesizer (Foster City, CA).

PCRB-1: 5'-ATCTGACTGGGAGAGACAAGTAG-3', a 23mer with its 5' end at nucleotide 6512 (antisense)

PCRB-2: 5'-GTTCTTTTAAGTCCTGTGCATC-3', a 23mer with its 5' end at nucleotide 6708 (sense)

PX1: 5'-TATCTCTAATAAACTGATC-3', a 19mer with its 5' end at nucleotide 6690 (sense)

PX2: 5'-AGTCCTGTGCATCAACTATCTCTAA-TATACTGA-3', a 35mer with its 5' end at nucleotide 6718 (sense)

PCRB-2 was used to prime reverse transcription reaction (RT). Both PCRB-1 and PCRB-2 were utilized for polymerase chain reaction (PCR). PX1 and PX2 were used in primer extension reaction. PX1 and PX2 were 5' end-labeled with [³²P]ATP (29). The reaction mixture (25 µl) consisting of 5 pmol of primer, 10 pmol of [γ ³²P]ATP (sp act 5,000 Ci/mmol, Amersham, Tokyo), 10 U T4 polynucleotide kinase (Takara Shuzo Co., Ltd., Kyoto), 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, and 10 mM 2-mercaptoethanol was incubated at 37°C for 1 h. Labeled oligonucleotides were precipitated in ethanol.

Assay of apoB mRNA editing

ApoB mRNA editing ratio was analyzed by RT-PCR coupled with dideoxynucleotide chain termination assay (30). Total cytosolic RNA was isolated in 4 M guanidine isothiocyanate through the cesium chloride cushion (31). Single-strand apoB cDNA was synthesized from 40 µg of crude RNA using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratory). The reaction mixture (50 µl) consisted of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10

1 mM dithiothreitol, 100 µg/ml BSA, 50 µg/ml actinomycin D (Sigma), 0.5 mM deoxynucleotide (dATP, dTTP, dGTP, dCTP), 2.4 µg primer (PCR-B-2), 20 U of RNasin (RNase inhibitor, Promega, Madison, WI), and 200 U/50 µl Moloney murine leukemia virus reverse transcriptase. Yeast tRNA (40 µg, Sigma) for negative control was added to the reaction mixture instead of rat RNA. After the incubation for 1 h at 37°C, cDNA and RNA were extracted with phenol-chloroform 1:1 (v/v). The cDNA region from nucleotide 6512 to 6718, corresponding to human apoB cDNA sequence, was amplified by PCR using primers PCR-B-1 and PCR-B-2 as described previously (32). The PCR-generated cDNA was treated by 5 µg of DNase-free RNase (Sigma) for breakdown of residual RNA and purified by passing through a Sephadex G-50 column (Boehringer-Mannheim, Mannheim, Germany). Then, primer extension was performed following the method of Hoeg et al. (33). In brief, 1/3 volume of PCR product was annealed to 0.3 fmol of 5'-³²P end-labeled PX1 or PX2 in a buffer (7 µl) containing 57 mM Tris-HCl, pH 7.5, 29 mM MgCl₂, 71 mM NaCl at 70°C for 10 min after denaturing at 95°C for 5 min. Then, the enzyme solution (3 µl) containing 1.6 U T7 DNA polymerase (United States Biochemical Co., Cleveland, OH), 1 mM dATP, dCTP, and dTTP, and 2.5 mM dideoxy GTP was added to the mixture, and incubated at 42°C for 10 min. Polymerization reaction of apoB-100-type cDNA was stopped at the position of 6666 (C); however, that of apoB-48-type cDNA was stopped at 6661 (C) but not at 6666(T). Elongated cDNA fragments were separated in 17% polyacrylamide gel and exposed to Kodak XOMAT AR film at -70°C for 30 min with an intensifying screen. To calculate the proportion of apoB-48 and apoB-100 cDNA, the bands corresponding to the apoB primer extension products were cut out from the gel and quantified by liquid scintillation counting in a Hewlett-Packard TriCarb 4530 liquid scintillation counter. Intra-assay CV of the ratio of apoB-48 type band/apoB-100 band signal intensity was 2.4%. PCR-generated cDNAs were ligated to BlueScript KS M13+ (Stratagene, La Jolla, CA) and transfected to DH5a strain *E. coli* cells. Nucleotide sequences of subcloned cDNA were determined by a previously described procedure (32). In order to confirm the results of primer extension assay, we sequenced several independent subclones and counted the proportion of apoB-100-type cDNA clones and apoB-48-type cDNA clones.

Northern blot and slot blot analyses of rat apoB mRNA

Northern blotting of rat apoB mRNA was performed by two methods previously described (32, 34). Total cytosolic RNA was suspended in 20 mM 3-[N-morpholino]propanesulfonic acid, pH 7.0, containing 1 mM

EDTA, 50% formamide, and 6.6% formaldehyde and denatured by heating at 80°C for 10 min. Total RNA was separated in 0.8% agarose gel and transferred to Genescreen (New England Nuclear, Boston, MA). A 3.0-kb rat apoB cDNA insert (rb9E) established by Dr. A. J. Lusis, University of California, Los Angeles, corresponding to the 3' coding region of the mRNA, was purchased from American Type Culture Collection (Rockville, Maryland, cat. no. 63109). A 1.9-kb rat β-actin cDNA (pAct-108) for internal standard was provided by Dr. K. O'Malley, Department of Anatomy and Neurobiology, Washington University, St. Louis, MO. The cDNA was labeled with ³²P by a random priming procedure. The filter was prehybridized in 50% formamide, 0.2% polyvinylpyrrolidone (mol wt 40,000, Sigma), 0.2% BSA (fraction V, Sigma), 0.2% ficoll (mol wt 400,000, Sigma), 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, and 125 µg/ml of denatured salmon sperm DNA at 42°C for 18 h, and hybridized to each of the ³²P-labeled cDNA probes (1–2 × 10⁶ cpm/ml) in the same buffer for 42°C for 24 h. After washing twice in 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 5 min at room temperature, followed by two washes in 2 × SSC, 1% SDS at 65°C for 30 min, and two washes in 0.1 × SSC at room temperature, the filters were exposed to Kodak X-OMAT X-ray film for 3 days at -70°C. Alternatively, rat mRNA isolated through an oligo(dT) column was subjected to 0.8% agarose gel electrophoresis and transferred onto nitrocellulose filters. Rat apoB mRNA was probed with a 26-bp synthetic oligodideoxynucleotide complimentary to rat apoB mRNA: 5'-GAGTTTGTGACAAATATGGGCATCAT-3'. The oligonucleotide was ³²P-radiolabeled using T4 polynucleotide kinase. For slot blot analysis of RNA, 2.5, 5, and 10 µg of RNA were blotted onto nitrocellulose filters and probed with ³²P-labeled rat apoB cDNA (rb9E).

Measurement of apoB secretion rate

ApoB secretion rate was measured by immunoprecipitation assay of liver tissue extract, using rabbit polyclonal antibodies against purified rat plasma apoB as described (35). Liver slices were washed with PBS at 37°C in a 50-ml conical tube, and 1 ml of warm methionine-deficient Dulbecco's modified Eagle's medium (DMEM) was added to each tube. After incubation for 30 min at 37°C in a 95% air, 5% CO₂, 1 mCi of [³⁵S]methionine was added to each tube, the medium was gently swirled, and the tubes were returned to the incubator for 30 min. After the medium was discarded, the tubes were washed with DMEM three times at 37°C, and 1 ml of DMEM containing excess cold methionine was added to each tube and incubated for 120 min at 37°C. After stopping the reaction on ice, the medium

was collected and centrifuged at 10,000 g for 20 sec. Five hundred microliter of supernatant was added into 1 ml of immunoprecipitation buffer containing anti-rat apoB IgG.

After a 24-h incubation on a rotating rack at 4°C, 30 μ l of Immunoprecipitin® (GIBCO BRL) was added, and incubations were continued for additional 30 min at room temperature. The suspension was then centrifuged at 10,000 g for 20 sec, the supernatant was discarded, and the pellet was washed six times with immunoprecipitation buffer containing 0.1% SDS, and once with 0.0625 M Tris-HCl, pH 6.8. The pellet was dissolved in 20 μ l of electrophoresis loading solution (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 0.001% bromophenol blue, and 10% glycerol) and boiled for 10 min. Samples were subjected to electrophoresis on SDS-3-10% polyacrylamide gradient gel at 25 w for 3 h. After electrophoresis, gels were fixed for 1 h, impregnated with ENHANCE® (NEN), dried, and exposed to X-ray film (XAR-5) for 2-7 days at -80°C.

Statistical analysis

Values were expressed as mean \pm standard deviation (SD). The significance of the difference between the mean values was evaluated by unpaired Student's *t*-test. $P < 0.05$ was considered as statistically significant.

RESULTS

Fasting plasma glucose and insulin concentrations were significantly higher in GK rats than those in control littermates as shown in Table 1 ($P < 0.001$ and $P < 0.01$, respectively). In GK + VMH rats, plasma glucose levels

were further increased to 19.2 mmol/L in average, although the mean IRI levels were decreased from 81 μ U/ml in GK to 53 μ U/ml ($P < 0.05$). Plasma cholesterol and triglyceride concentrations were increased in GK rats ($P < 0.01$ and $P < 0.05$, respectively) and again were further elevated in GK + VMH. Food intakes of VMH, GK, and GK + VMH rats were increased by 20%, 30%, and 30%, respectively, compared with controls (data not shown).

Plasma lipoprotein and apoB levels are also shown in Table 1. Plasma VLDL-triglyceride concentrations were increased in GK and GK + VMH rat compared with those in controls ($P < 0.05$ and $P < 0.001$, respectively) (Table 1). There were no significant differences in VLDL-cholesterol levels among the four groups. LDL-cholesterol concentrations were significantly increased in VMH, GK, and GK + VMH rats compared with controls ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively). HDL-cholesterol levels were significantly increased only in GK + VMH rats compared with controls ($P < 0.01$).

Plasma apoB-100 levels quantified by immunoblot were significantly higher only in GK + VMH rat by 47% than those in controls (Table 1). ApoB-48 levels were more markedly increased in GK and GK + VMH by 239% and 470% ($P < 0.01$) respectively, in comparison with controls. Therefore, apoB-48/apoB-100 ratios were significantly increased in GK and GK + VMH rat compared with those in controls (Fig. 1). The apoB-48/apoB-100 ratio in VMH rat showed a minimal increase.

ApoB mRNA editing was evaluated in the liver and small intestine of the rats under study. PCR products of rat apoB mRNA were about 200 bp in size, consistent with the predicted sizes (205 bp). No DNA band was

TABLE 1. Plasma glucose, immunoreactive insulin, lipid, lipoprotein, and apolipoprotein B levels

	Control	VMH	GK	GK + VMH
Number	5	6	5	6
Body weight (g)				
9 weeks	293 \pm 13	302 \pm 24	290 \pm 8	298 \pm 14
25 weeks	351 \pm 22	506 \pm 97 ^c	411 \pm 24 ^a	376 \pm 36
Glucose (mmol/L)	6.4 \pm 0.5	6.7 \pm 0.5	10.2 \pm 1.2 ^c	19.2 \pm 1.9 ^c
IRI (μ U/ml)	30 \pm 6	48 \pm 14 ^a	81 \pm 25 ^a	53 \pm 10 ^b
Triiodothyronine (nmol/L)	18.4 \pm 1.3	17.8 \pm 3.9	16.0 \pm 1.4	15.7 \pm 3.7
Total cholesterol (mmol/L)	1.58 \pm 0.07	1.81 \pm 0.13 ^b	1.97 \pm 0.10 ^b	2.17 \pm 0.18 ^a
Triglyceride (mmol/L)	0.41 \pm 0.08	0.52 \pm 0.13	0.55 \pm 0.07 ^a	0.84 \pm 0.12 ^a
VLDL-TG (mmol/L)	0.36 \pm 0.06	0.46 \pm 0.10	0.48 \pm 0.05 ^a	0.81 \pm 0.16 ^c
VLDL-CH (mmol/L)	0.11 \pm 0.02	0.10 \pm 0.02	0.10 \pm 0.02	0.15 \pm 0.04
LDL-CH (mmol/L)	0.29 \pm 0.02	0.74 \pm 0.22 ^b	0.52 \pm 0.05 ^c	0.50 \pm 0.07 ^c
HDL-CH (mmol/L)	1.17 \pm 0.07	0.96 \pm 0.18	1.37 \pm 0.31	1.54 \pm 0.20 ^b
ApoB-100 (arbitrary unit)	44.7 \pm 12.0	48.8 \pm 12.9	53.5 \pm 15.1	65.9 \pm 14.6 ^a
ApoB-48 (arbitrary unit)	7.0 \pm 2.2	10.3 \pm 3.1	23.7 \pm 8.5 ^b	39.9 \pm 14.8 ^b
ApoB-48/apoB-100 Ratio	0.15 \pm 0.03	0.21 \pm 0.01 ^a	0.44 \pm 0.08 ^c	0.60 \pm 0.17 ^c

Values are expressed as mean \pm standard deviation (SD). Values (except for body weight) were obtained at the age of 25 weeks. ApoB-100 and apoB-48 levels are shown as arbitrary units/20 μ l of plasma in immunoblot analysis. Abbreviations: CH, cholesterol; TG, triglyceride.

^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ (versus control).

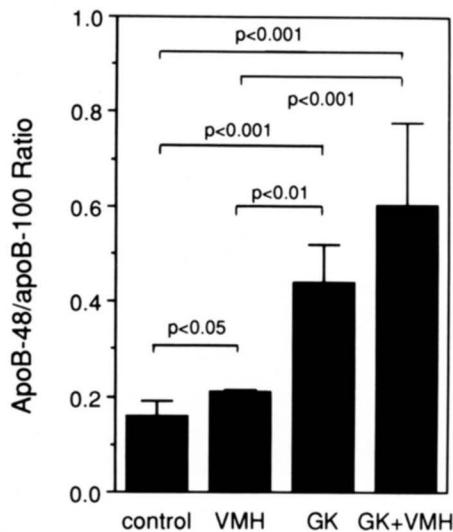


Fig. 1. ApoB-48/apoB-100 ratios in whole plasma quantified by Western blot. Delipidated proteins from whole plasma were subjected to 3–6% gradient SDS polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membranes and incubated with rabbit anti-rat apoB IgG, followed by incubation with peroxidase-conjugated anti-rabbit IgG. Each band was colorized by hydrogen peroxide and 3,3'-diaminobenzidine tetrahydrochloride dihydrate. ApoBs were identified by molecular weight makers, myosin heavy chain (molecular weight 200 kD), β -galactosidase (116 kD), and phosphorylase B (95.5 kD) and parallel electrophoresis of apoBs from plasma VLDL and lymph chylomicrons. Lanes 1–4 show results from control, ventromedial hypothalamus-lesioned (VMH), GK, and GK + VMH rats, respectively. Error bars show one standard deviation.

seen when yeast tRNA was used as a template (data not shown). In order to quantify the relative proportion of apoB-100- and apoB-48-type mRNA, primer extension was carried out in the presence of dideoxyguanosine GTP. In this system, primer PX1 annealed to the apoB-100 cDNA was extended to the C at nucleotide 6666 and generated a product of 25 nucleotides (nt). Primer PX1 annealed to apoB-48 cDNA was extended to the next upstream C and generated a product of 30 nt (Fig. 2). Primer PX2 annealed to apoB-100 and B-48 cDNAs generated extension products of 43 nt and 48 nt, respectively. We determined the proportion of apoB-48-type mRNA to apoB-100-type mRNA in GK and GK + VMH rats that showed a higher ratio of apoB-48/apoB-100 peptides in plasma compared to those in controls. ApoB-48/apoB-100 cDNA ratio (TAA/CAA ratio) was significantly higher in the liver from GK and GK + VMH rats than in control liver as shown in Fig. 3 ($P < 0.01$). There was no significant difference in TAA/CAA ratio between control and VMH rats or between GK and GK + VMH rats. These findings indicate that enhancement of hepatic apoB mRNA editing exists in the NIDDM rats. However, the development of VMH lesion had no additional effect on hepatic apoB mRNA editing. The experi-

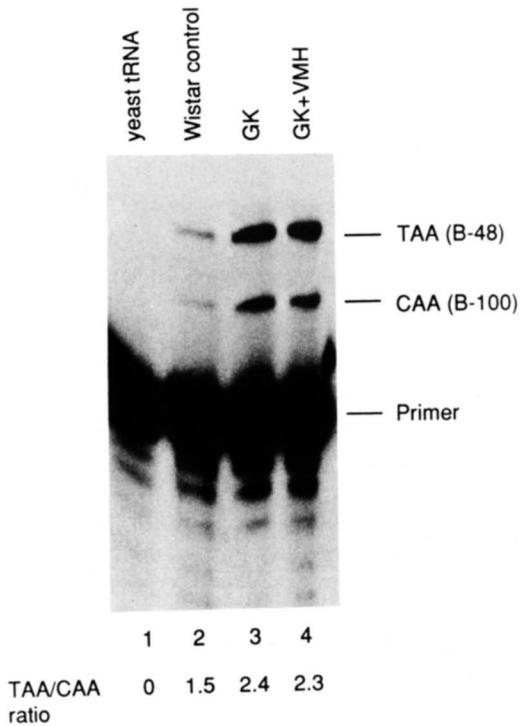


Fig. 2. ApoB mRNA editing detected by primer extension analysis in the liver of GK rats. Oligonucleotide primer PX1 annealed to apoB cDNA generated by reverse transcriptase–polymerase chain reaction from total RNA (40 μ g) was elongated by DNA polymerase in the presence of dATP, dCTP, dTTP, and dideoxyguanosine GTP. Products were subjected to 17% polyacrylamide gel electrophoresis. The gel was exposed to autoradiography. TAA (apoB-48-type cDNA)/CAA (apoB-100-type cDNA) ratio was quantified by liquid scintillation counting of each band cut out from the gel (numbers at bottom).

ment using PX2 as the extension primer confirmed these results (data not shown). ApoB cDNA signal for CAA codon in the intestine was only ~5% of that for TAA codon. There was no significant change in the ratio of apoB-48-type cDNA to apoB-100-type cDNA in the intestines among the four groups. As shown in Fig. 4, sequencing analysis demonstrated no base transition except for the C to T at the position of 6666 among the 205 bases of PCR-amplified cDNA region. There was no mutation in apoB mRNA from nt 6512 to nt 6708 in GK rats. To confirm the data from primer extension analysis, several subclones were sequenced. The ratio of apoB-48/apoB-100 clones was 16/6 (= 2.7) in GK and 13/8 (= 1.6) in control, which was consistent with the results of primer extension assay.

Signal intensity of hepatic apoB mRNA on Northern blot was apparently increased in VMH rat compared with control rat, when a 3.0-kb rat apoB cDNA insert, rb9E, or the synthetic oligonucleotide was used as the primer. Figure 5A shows a Northern blot of rat apoB mRNA. The amount of apoB mRNA in the liver was

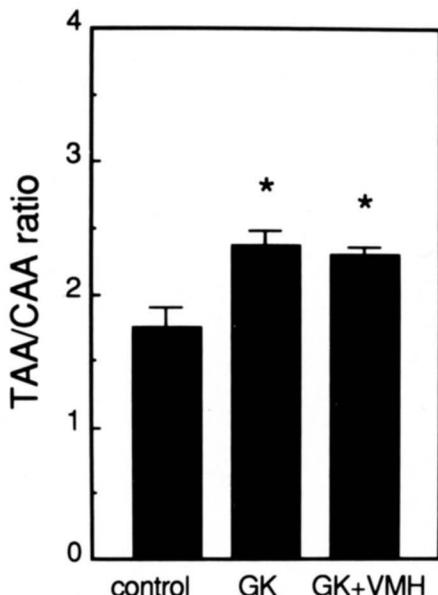


Fig. 3. TAA (apoB-48-type cDNA)/CAA (apoB-100-type cDNA) ratio in RT-PCR generated products. The number of animals is four in each group. Assay was carried out in triplicate. PX1 was used as the primer. * $P < 0.05$ (versus control).

quantified by slot blot analysis, using rat apoB cDNA, rb9E. ApoB mRNA level in VMH rat was higher by ~50% than in control (Fig. 5B), which was consistent with the data reported previously by our group (34). However, there was no significant difference in the amount of hepatic apoB mRNA between GK and control groups.

In order to further clarify the discrepancy between apoB-48/B-100 mRNA ratio and plasma apoB-48/B-100 protein ratio, we measured the ratio of apoB-48/B-100 protein secretion rate by immunoprecipitation assay using the liver specimen. As shown in Fig. 6, the ratio of apoB-48/B-100 protein secretion rate was increased by 10% in GK rats in comparison with controls ($P < 0.05$). These data suggested that an enhancement of apoB mRNA editing might contribute to the increase of apoB-48 protein secretion in the NIDDM rat.

DISCUSSION

In the present study, we observed that plasma apoB-48 levels in GK rats were increased and that both hepatic apoB mRNA editing and apoB-48 protein secretion rate were enhanced compared with controls. However, the extent of both apoB mRNA editing and protein synthesis was less than expected from the data of plasma protein levels (40% increase in mRNA ratio and 10% increase in protein synthetic rate versus 240% in plasma protein ratio). As apoB secretion was reported to be

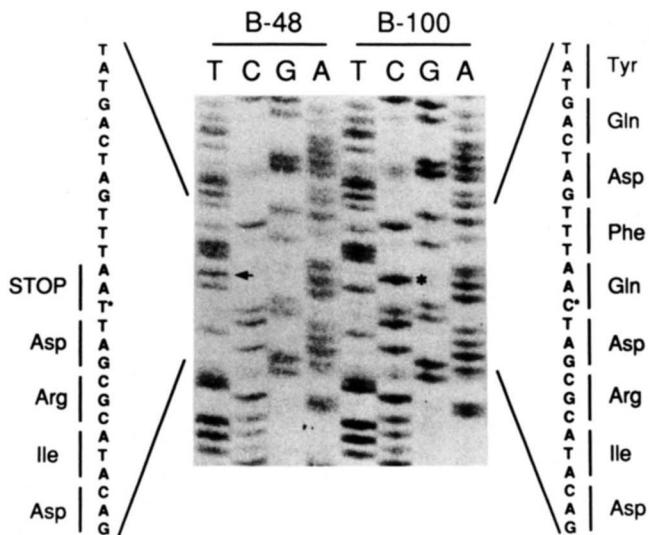
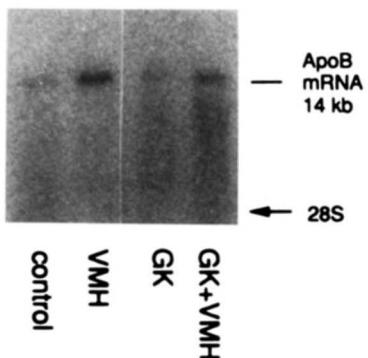


Fig. 4. Nucleotide sequences of apoB cDNAs around the 6666 nucleotide position in the liver of GK rat. PCR-generated cDNA fragments were ligated to BlueScript vector and introduced to DH5a cells. Nucleotide sequence of subcloned cDNA was determined by the dideoxy sequencing method.

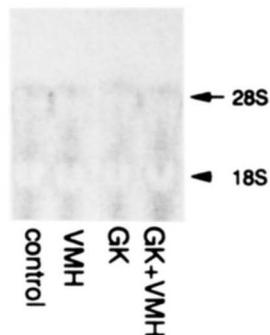
regulated at posttranscriptional protein stability (36), apoB mRNA editing may not directly enhance apoB protein secretion. We previously reported an enhancement of intestinal acyl-CoA:cholesterol acyltransferase activities in diabetic animals (2, 3). The increase of the plasma apoB-48 level might be affected by elevated exogenous lipoprotein secretion through intestinal cholesterol absorption. Furthermore, decreased lipoprotein lipase activity could also lead to the accumulation of chylomicron remnants in plasma and subsequently to the marked increase of the plasma apoB-48 level. Our current findings indicate that increased hepatic apoB mRNA editing could partly, but not exclusively, contribute to the increase of apoB-48 levels in the NIDDM rats.

The mechanism of enhanced apoB mRNA editing in GK rats is unknown at the present time. As GK rats are a genetic mutant with glucose intolerance, it is possible that apoB gene mutation may also be present in this model. If a mutation occurs near the consensus sequence (26 bases) required for apoB mRNA editing, the mRNA editing could be influenced (37–39). However, there was no mutation between nucleotide positions 6512 and 6708. Plasma thyroid hormone was shown to enhance the mRNA editing in the hepatocytes (19). Moreover, it is generally accepted that low plasma triiodothyronine level is associated with diabetes mellitus. However, according to our results, free triiodothyronine levels were not significantly reduced in the GK rats. Therefore, thyroid hormone is not a likely candidate factor causing the enhancement of apoB mRNA editing in this model.

A. Northern blot of apoB



B. Acridine orange stain



C. Slot blot analysis

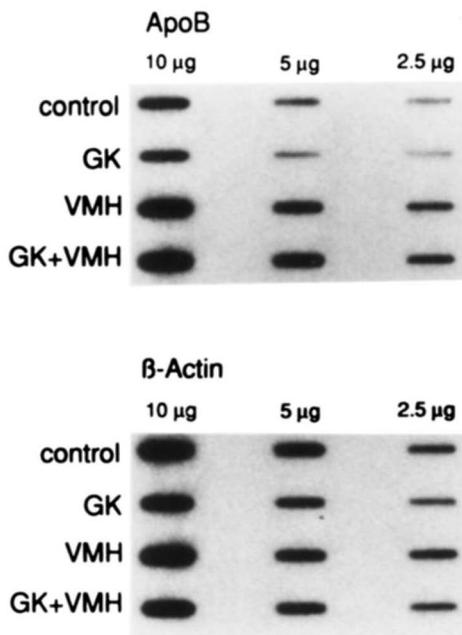


Fig. 5. Northern and slot blot analyses of rat apoB mRNA. Ten μ g of total RNA from rat liver was separated by 0.8% agarose gel electrophoresis, transferred to GeneScreen[®] filter, and probed with a 3.0-Kb 32 P-labeled rat apoB cDNA insert (panel A). Ribosomal RNA (28S) is indicated by an arrow. Panel B shows acridine orange-stained gel. For slot blot, different amounts of liver RNA (2.5, 5, and 10 μ g) were applied onto nitrocellulose filters and hybridized with 32 P-labeled rat apoB cDNA or rat β -actin (panel C).

Pullinger et al. (36) reported that insulin slightly suppressed the transcription of apoB gene in a human hepatoma cell line, HepG2 cells. Recently, Thorngate et al. (40) reported that insulin stimulated the biosynthesis and secretion of apoB-48 in rat hepatocytes by regulating apoB mRNA editing. In the present study, we are not able to conclude that the increase of plasma insulin level could lead to an enhanced apoB mRNA editing in the liver. GK rats with increased plasma insulin levels showed an enhanced apoB mRNA editing in comparison with controls. However, GK + VMH rats that have lower insulin levels than GK rats showed the same editing activity as GK rats. Furthermore, in our preliminary study we observed that hepatic apoB mRNA editing activity was not changed in streptozotocin-induced diabetic rats, an insulinopenic diabetic model (data not shown). This observation was similar to the data published by Sparks et al. (41). ApoB secretion rate is regulated by posttranscriptional protein stability, and insulin influences not only apoB mRNA editing but also apoB

secretion. In the current study, the increment of apoB-48/B-100 protein secretion ratios in the liver of GK rats was lower than that of apoB mRNA editing ratio. Both streptozotocin-induced rats and VMH-lesioned rats are experimental animal models that have similar apoB mRNA editing activity to control rats. As the GK rat is a genetically determined NIDDM model, some genetic factors might be involved in the enhancement of apoB mRNA editing in the liver.

The predominant apoB form secreted from the human liver is apoB-100. However, according to the reports by Levy et al. (13) and Hoeg et al. (33), human intestines synthesize and secrete not only apoB-48 but also apoB-100. Furthermore, both apoB-100- and apoB-48-type mRNAs are identified in human intestinal mucosa (42). These findings suggest that apoB-100-containing lipoproteins are also secreted from human intestine. If it is the case, the proportion of apoB-48 to apoB-100 secreted from the intestine could be modified in diabetic humans as shown in the liver of GK rats. Investi-

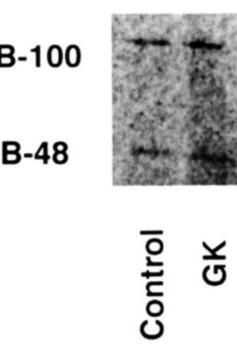
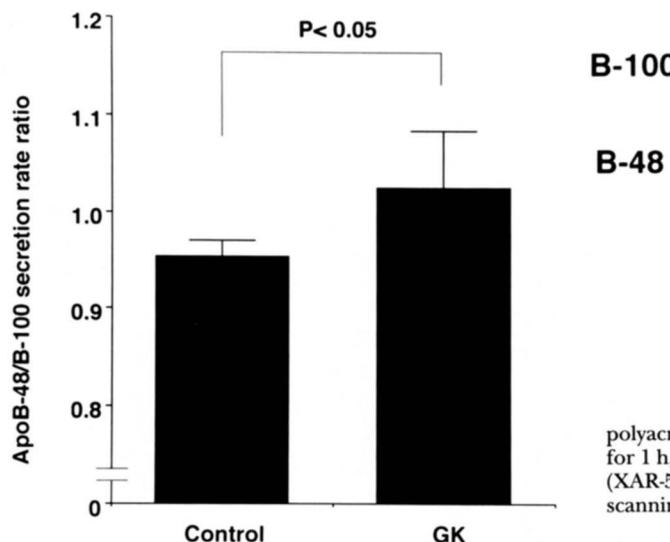


Fig. 6. ApoB-48/B-100 secretion rate ratio in control and GK rats by immunoprecipitation analysis. Liver slices were washed in a 15-ml conical tube with PBS, and 1 ml of methionine-deficient DMEM was added to each tube. After 30 min incubation, 1 mCi of [³⁵S]methionine was added to each tube, the medium was swirled and returned to the incubator for 30 min. Pulse-chase study was performed at 37°C for 120 min, after which immunoprecipitation buffer containing anti-rat apoB IgG was added. After a 24-h incubation on a rotating rack at 4°C, 30 µl of Immunoprecipitin® (GIBCO BRL) was added and incubations were continued for 30 min at room temperature. The suspension was then centrifuged, the supernatant was discarded, and the pellet was washed with immunoprecipitation buffer. Samples were subjected to electrophoresis on SDS-3-10% polyacrylamide gradient gel at 25 w for 3 h. After electrophoresis, gels were fixed for 1 h, impregnated with ENHANCE® (NEN), dried, and exposed to X-ray film (XAR-5) for 2-7 days at -80°C. Each band was quantitated by densitometric scanning.

gation of hormonal, nutritional, and pharmacological factors modifying apoB mRNA editing might provide new insights into the pathogenesis of dyslipoproteinemia in diabetics.

In conclusion, elevated levels of plasma apoB-48 were shown in the NIDDM rats with hyperglycemia and insulin resistance, in which an enhanced hepatic apoB mRNA editing was demonstrated. Molecular basis of enhanced mRNA editing in the GK rat remains to be elucidated in future studies. ■

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