Atherogenic dyslipidemia and altered hepatic gene expression in SHRSP.Z-Lepr^{fa}/IzmDmcr rats

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Abstract. We investigated lipid and lipoprotein abnormalities in SHRSP fatty rats as a new animal model of metabolic syndrome. We examined differentially expressed genes in the liver, one of the major tissues contributing to lipid metabolism. Using gel filtration high performance liquid chromatography, increased cholesterol concentrations of small particle size low-density lipoprotein (LDL) fractions were observed in SHRSP fatty rats, whereas the Zucker Fatty strain did not show a similar elevation of cholesterol content. Existence of apolipoprotein B in these fractions was confirmed by Western blotting. The small particle size of the LDL fractions was significantly decreased by a 4-week fenofibrate treatment. Microarray analysis identified seventeen genes that were significantly upregulated and ten that were significantly decreased in liver tissues of SHRSP fatty rats compared with levels in SHRSP rats. Stearoyl-coenzyme A desaturase 1, fatty acid synthase, ATP citrate lyase, and sterol regulatory element binding factor 1 genes were among the upregulated genes. These findings suggest that SHRSP fatty rats carry small dense LDL like particles which is a common lipid abnormality in the metabolic syndrome. Three of ten genes upregulated in liver tissues of SHRSP fatty rats play a role in this metabolic abnormality and are a therapeutic target of this metabolic syndrome.

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Introduction

Metabolic syndrome is characterized by a constellation of cardiovascular risk factors, including atherogenic dyslipidemia, abnormal glucose tolerance, hypertension, and visceral obesity, which are intimately associated with insulin resistance and hyperinsulinemia (1-3). The dyslipidemia of metabolic syndrome features hypertriglyceridemia involving elevated concentrations of triglyceride rich lipoproteins, subnormal levels of high-density lipoprotein (HDL) choresterol, or both. Major quantitative modifications of the atherogenic lipid profile typically include a small, dense, low-density lipoprotein (sdLDL) phenotype (4-6). Retrospective studies provided evidence that the preponderance of sdLDL particles is associated with an increased cardiovascular risk, a suggestion that was later also supported by a prospective study (7). These findings imply that the evaluation of sdLDL cholesterol levels enhances the prediction of cardiovascular events (8,9).

Hiraoka-Yamamoto *et al* (10) established a new rat model of metabolic syndrome, SHRSP.Z-Lepr^{fa}/IzmDmcr, by crossing SHRSP rats of the Izumo strain (a genetic model of severe hypertension) with Zucker Fatty (ZF) rats. SHRSP fatty rats carry the leptin receptor *OB-Rb* gene mutation found in ZF rats and become obese while developing hypertension. We previously reported that SHRSP fatty rats exhibit obesity and hypertension accompanied by hypertrophy of the midlayer smooth muscle of the arteries, increased non-fasting triglyceride levels and increased insulin resistance. Therefore, we determined that the phenotype of SHRSP fatty rats is similar to that of human metabolic syndrome and is a useful tool for investigating the molecular mechanisms underlying metabolic syndrome (11).

Fibric acid derivatives (fibrates) are lipid-lowering drugs which selectively target therapeutic goals for individuals with features of metabolic syndrome. One specific fibrate, fenofibrate, was effective in normalizing lipoprotein levels and reducing insulin resistance in patients with metabolic syndrome (12,13). Additionally, plasma triglyceride lowering agents enlarge the LDL size (14). However, few studies examine the quantitative change of LDL sub-species by fibrate treatment. One of these studies, by Takuno *et al* (15), reported that fenofibrate decreased sdLDL specifically, without lowering total LDL levels.

The liver is a major determinant of the whole body fatty acid and neutral lipid metabolism as well as circulating levels of atherogenic apolipoprotein B containing lipoproteins (16-18). A marked increase in the production of apolipoprotein B containing lipoprotein in the liver is frequently associated with metabolic syndrome (19). In fact, Adiels *et al* reported that overproduction of very low density lipoprotein (VLDL) particles is driven by the amount of fat accumulated in the liver (20). However, underlying mechanisms for this overproduction of fatty liver related lipids is still unclear.

In the current study, to clarify the mechanism of such lipid abnormalities in metabolic syndrome, we examined the plasma lipoprotein profile, evaluated the effect of fenofibrate, and examined the liver gene expression profile in SHRSP fatty rats.

Materials and methods

Animal subjects. Our study conforms to the guidelines published in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Wistar-Kyoto/Izumo (WKY) and SHRSP/Izumo rats were obtained from Disease Model Cooperative Research Association (Kyoto, Japan). ZF rats were obtained from Tokyo Experimental Animal (Tokyo, Japan). SHRSP fatty were obtained by crossing SHRSP/Izumo with ZF rats as described previously (10). Four of each strain of rats were fed standard rat chow and then fasted for 12 h before blood collection.

Fenofibrate (100 mg/kg) (Sigma-Aldrich, Irvine, UK) was dissolved/suspended in 0.25% methylcellulose (Wako, Osaka, Japan) at a concentration that would allow daily doses of 10 ml suspended compounds. After a 4-week fenofibrate treatment of four SHRSP fatty rats, the animals were euthanized by diethyl ether and blood was collected by cardiac puncture.

Histologic examination. Twenty-four week-old WKY, SHRSP, ZF, and SHRSP fatty rats were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg IP; DS Pharma Biomedical, Osaka, Japan) and perfused with saline followed by 10% neutral buffered formalin. The liver was removed, tissue specimens were embedded in paraffin, and 2-μm thick slices were stained with hematoxylin-eosin. These procedures were performed by a pathologist with no prior knowledge of the experimental groups.

High-performance liquid chromatography (HPLC). Plasma lipoprotein profiles were analyzed by HPLC using gel-permeation column(s) (Lipopropak XL; 7.8 mm x 300 mm; Tosoh, Tokyo, Japan) with 0.05 M Tris-buffered acetate, pH 8.0, containing 0.3 M sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 ml/min and an online enzymatic lipid-detection system (21-23).

Western blot analysis for apolipoprotein B and AI proteins. Fractionated samples were incubated with equal amount of lysis buffer (6% SDS, 40% glycerol, 0.5% bromophenol blue) at 95˚C for 5 min, subjected to 2-15% SDS-polyacrylamide gel (Daiichi Chemical, Tokyo, Japan) electrophoresis, and electro-blotted onto PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ). Blots were incubated with goat polyclonal antibody specific for anti-apolipoprotein B or rabbit polyclonal antibody specific for apolipoprotein AI (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies, and then with rabbit anti-goat IgG (Cappel, West Chester, PA) or goat anti-rabbit IgG (RPN2124, GE Healthcare), as secondary antibodies. Bound antibodies were detected by enhanced chemiluminescence (RPN2124, GE Healthcare) and scanned by lumino-image analyzer (LAS-3000, Fuji film, Tokyo, Japan).

DNA microarray procedure. Total RNA was obtained from liver tissue of SHRSP fatty and SHRSP rats by successive extractions with Trizol and RNeasy Mini Kits (Qiagen, Valencia, CA). The RNA was assessed for quality and quantity with a Bioanalyzer (Agilent, Palo Alto, CA). DNA microarray analysis was performed according to the manufacturer's instructions (Affimetrix, Santa Clara, CA). In brief, double-stranded cDNA was synthesized from 10 μg of total RNA by reverse transcription with SuperScript Choice System (Invitrogen, Carlsbad, CA). Biotinylated cRNA was transcribed from the double-stranded cDNA by T7 RNA polymerase reaction with an RNA Transcript Labeling Kit (Enzo Biochem, Framingdale, NY), fragmented, and applied to Gene Chips (Rat Genome 230 2.0 Array, Affimetrix). After hybridization for 16 h at 45˚C, the Gene Chip was washed and labeled with R-phycoerythrin streptavidin using the Affymetrix Fluidics Station 400. The fluorescent signal intensities were measured with an Affymetrix Scanner. Raw data were extracted with Microarray Suite 5 (Affymetrix) and analyzed with GeneSpring GX (Agilent). Values below 0.01 were set to 0.01, and each measurement was divided by the 50th percentile of all measurements in that sample. Each gene was divided by the median of its measurements in all samples. If the median of the raw values was <10 then each measurement for that gene was divided by 10 if the numerator was >10, otherwise the measurement was disregarded. Results were expressed as a fold change of the mean of four SHRSP fatty and four SHRSP rats.

Statistical analysis. Results are given as the mean ±SEM. The significance of differences between the mean values was evaluated by Student's t-test for unpaired data. Differences of $P<0.05$ were considered significant.

Results

Body weight, plasma lipid, and lipoprotein profile. Body weight of SHRSP fatty rats was significantly higher compared to WKY rat strain, and almost comparable with the ZF strain (Table IA). Plasma total cholesterol level and cholesterol levels in each major lipoprotein fraction are shown in Table IB. Plasma total cholesterol level in SHRSP fatty rats (181.2±8.4 mg/dl) was significantly higher than that in each parental strain ($p<0.05$). Whereas no significant difference was observed in LDL fraction

Table I. Plasma total cholesterol and triglyceride concentrations of each fraction of SHRSP fatty, ZF, and SHRSP rats.

A, Body weight (grams)	
SHRSP fatty	446.0±22.0[a]
ZF	452.0±8.0[a]
SHRSP	262.0±4.9

B, Total cholesterol	Total	CM (>80 nm)	VLDL (30-80 nm)	LDL (16-30 nm)	HDL (8-16 nm)
SHRSP fatty	181.1±8.4[a]	0.2±0.0	48.6±3.2[a]	31.3±3.6[a]	101.0±4.1[a]
ZF	97.8±3.4	0.0±0.0	9.7±0.5	8.3±0.7	79.8±2.3
SHRSP	67.9±2.0	0.0±0.0	2.5±0.6	9.0±0.3	56.4±2.7
C, Triglycerides					
SHRSP fatty	862.7±43.6[a]	4.4±0.5	747.7±42.5[a]	74.0±1.2[a]	36.5±0.8[a]
ZF	231.0±10.2[a]	0.8±0.1	196.6±8.8[a]	21.2±1.0	12.5±1.1
SHRSP	39.2±8.6	0.1±0.0	23.7±7.7	11.6±0.8	3.8±0.3

mg/dl; [a]p<0.05 vs SHRSP.

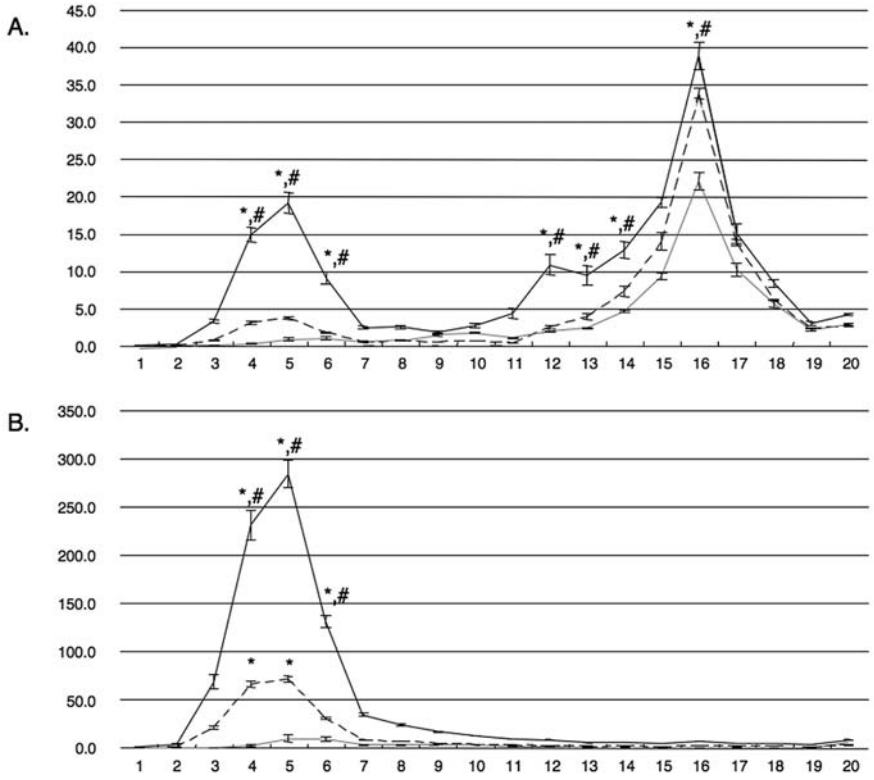

Figure 1. Lipoprotein profile of each rat strain determined by high performance liquid chromatography. (A) Elution pattern of HPLC monitored by cholesterol concentration in each fraction. (B) Elution pattern of HPLC monitored by triglyceride concentration in each fraction. Black solid line, lipid concentration in each fraction from SHRSP fatty; dashed line, lipid concentration in each fraction from ZF; solid line, lipid concentration in each fraction from SHRSP.

cholesterol levels, cholesterol level of HDL fraction in SHRSP fatty rats was significantly higher (101.01±4.05 mg/dl) than in each parental strain (p<0.05) (Table IB). Plasma triglyceride levels in SHRSP fatty rats were significantly higher (862.7±43.6 mg/dl) compared to the parental strains (p<0.05), and in SHRSP fatty rats, about 86.7% of plasma triglycerides were included in the VLDL fraction (747.7±42.5 mg/dl) (Table IC).

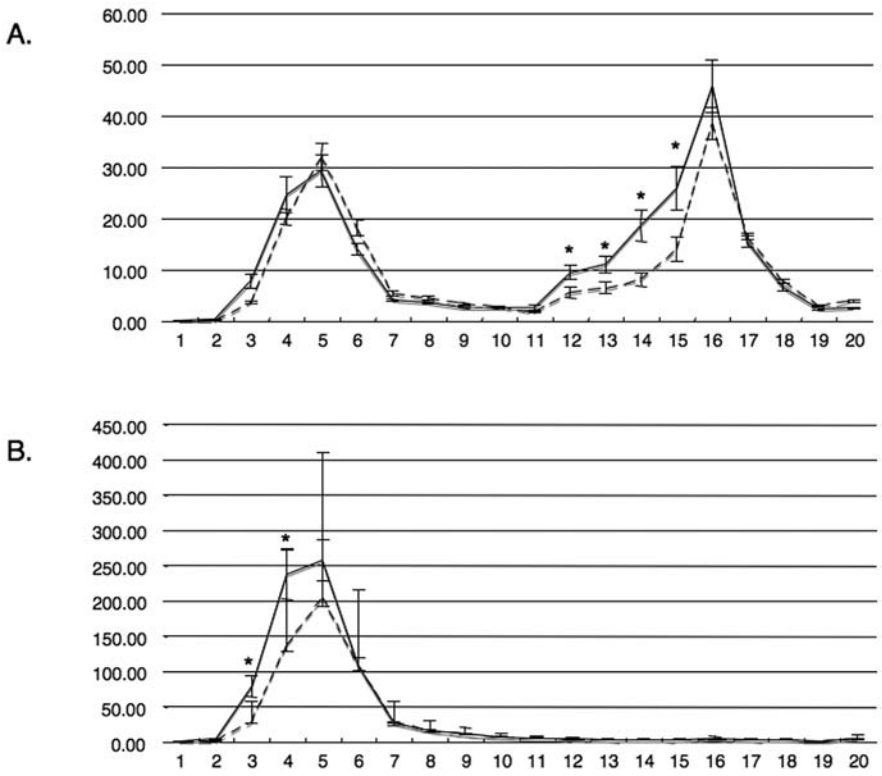

Figure 2. Effect of fenofibrate administration on lipoprotein profile of SHRSP fatty determined by high performance liquid chromatography. (A) Elution pattern of HPLC monitored by cholesterol concentration in each fraction. (B) Elution pattern of HPLC monitored by triglyceride concentration in each fraction. Solid line, lipid concentration of each fraction before fenofibrate treatment; dashed line, lipid concentration in each fraction after fenofibrate treatment.

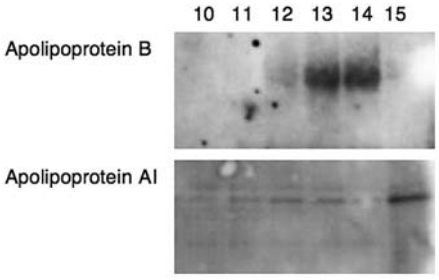

Figure 3. Western blot analysis for apolipoprotein B and AI proteins in samples from HPLC fractions. Fraction numbers 10 to 15 are identical to fraction numbers in Figs. 1 and 2.

Lipoprotein profile of each rat strain determined by HPLC. To determine the lipoprotein profile of each rat strain, gel filtrated HPLC was performed and cholesterol (Fig. 1A) and triglyceride (Fig. 1B) levels in each fraction were evaluated. In VLDL fractions (fractions 3-7), cholesterol and triglyceride levels in SHRSP fatty rats were significantly higher than in each parental strain. In fractions 12, 13, and 14, significantly higher cholesterol levels were also observed in SHRSP fatty rats, indicating the existence of very small particle size LDLs. In fraction 16, cholesterol levels were significantly lower in SHRSP rats compared with SHRSP fatty or ZF rats.

Western blot analysis for apolipoprotein B and AI proteins. To examine the feature of lipoproteins included in fractions 12, 13, and 14, we performed Western blot analysis for apolipoprotein B and AI on each fraction from SHRSP fatty rats. Apolipoprotein B was detected by Western blot analysis in fractions 12, 13, and 14, indicating the existence of apolipoprotein B containing small particle size lipoprotein (Fig. 3). Apolipoprotein AI was strongly stained in the sample from fraction 15, indicating the existence of large particle size HDL (Fig. 3).

Effect of fenofibrate on lipid and lipoprotein profiles of SHRSP fatty rats. Plasma lipid and lipoprotein profile before and after fenofibrate administration are shown in Table II. Plasma triglyceride levels were significantly reduced after 4 weeks of fenofibrate administration. VLDL levels also showed a tendency to lower after fenofibrate treatment. However, the results were not statistically significant.

The effect of fenofibrate on the plasma lipoprotein profile was evaluated by HPLC. Cholesterol levels in fractions 12, 13, and 14 were significantly reduced after fenofibrate treatment and triglyceride levels in fractions 3 and 4 were significantly reduced after fenofibrate treatment.

Pathologic liver findings in SHRSP fatty, SHRSP, and ZF rats. The typical hematoxylin-eosin staining results obtained upon histological examinations are shown in Fig. 4. The liver in SHRSP fatty and ZF rats showed marked lipid accumulation compared to that of SHRSP rats.

Gene expression profiling of liver tissue by DNA microarray. To identify the genes differentially expressed (more than 3-fold) between SHRSP fatty and SHRSP rats, we carried out microarray analysis. Seventeen genes were significantly up-

Table II. Plasma total cholesterol and triglyceride concentrations before and after fenofibrate administration.

	Total	CM (>80 nm)	VLDL (30-80 nm)	LDL (16-30 nm)	HDL (8-16 nm)
Total cholesterol					
Before	227.7±18.8	0.31±0.1	79.3±9.2	31.4±3.7	116.7±13.9
After	194.3±15.2	0.2±0.0	79.1±6.2	24.0±3.0	91.1±7.7
Triglycerides					
Before	777.0±99.8[a]	5.2±1.1	707.2±90.4	45.6±6.2	19.0±2.3
After	566.3±36.6	1.2±0.2	507.5±33.5	40.2±2.7	17.5±0.7

mg/dl; [a]p<0.05 vs SHRSP.

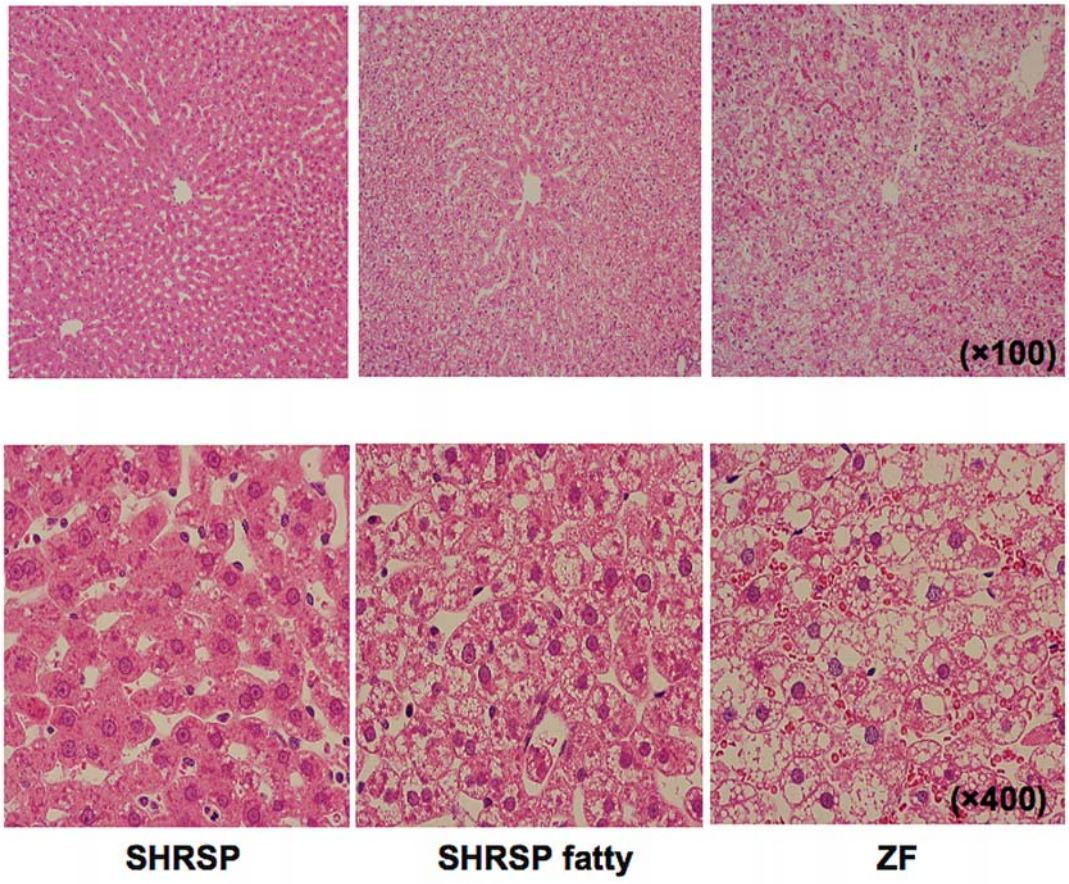

Figure 4. Pathologic findings of liver in SHRSP, SHRSP fatty, and ZF rats.

regulated in liver tissue in SHRSP fatty rats compared to SHRSP rats, including stearoyl-coenzyme A desaturase 1, fatty acid synthase, alcohol dehydrogenase 1, glucokinase, and sterol regulatory element binding factor 1.

Ten genes were significantly reduced (less than 0.3-fold) in the liver tissue of SHRSP fatty rats compared with SHRSP rats, inlcuding Fatty acid desaturase 1, ATP-binding cassette, subfamily C, member 2, solute carrier organic anion transporter family, member 1b2, and Hydroxysteroid (17-ß) dehydrogenase 2. Results for all the differentially expressed genes are in Table III. Changes in gene expression in SHRSP fatty rats compared with the control strains discovered by microarray analysis were further confirmed by RT-PCR.

Discussion

In this study, we investigated lipid and lipoprotein abnormalities in SHRSP fatty rats as a new animal model of metabolic syndrome and examined the differentially expressed genes in the liver, since the liver is one of the major contributors to lipid metabolism.

In a previous study, we found that SHRSP fatty rats exhibited obesity and hypertension accompanied by hypertrophy of the midlayer smooth muscle of the arterioles, increased non-fasting triglyceride levels, and increased insulin resistance. Additionally, we determined that the phenotype of SHRSP fatty rats is similar to that of human metabolic

Table III. Genes differentially expressed in liver tissue of SHR-SP fatty and SHRSP.

Fold change	Description
Upregulated genes	
25.66[a]	Stearoyl-coenzyme A desaturase 1
11.77[a]	Fatty acid synthase
5.32[a]	Thyroid hormone responsive protein
4.56[a]	Ethylmalonic encephalopathy 1
4.52[a]	Alcohol dehydrogenase 1
3.89[a]	Flavin containing monooxygenase 5
3.79[a]	Glucokinase
3.51[a]	Rat senescence marker protein 2A gene, exons 1 and 2
3.42[a]	ATP citrate lyase
3.32[a]	Cytochrome P450, family 4, subfamily A, polypeptide 22
3.23[a]	Serine dehydratase-like
3.19[a]	Cytochrome P450, family 2, subfamily b, polypeptide 2
3.17[a]	Glutamate oxaloacetate transaminase 1
3.11[a]	Glycerol-3-phosphate acyltransferase, mitochondrial
3.08[a]	Similar to mKIAA1002 protein
3.06[a]	Sterol regulatory element binding factor 1
3.02[a]	Centaurin, γ3
Downregulated genes	
0.07[a]	Serine protease inhibitor
0.08[a]	Cysteine sulfinic acid decarboxylase
0.09[a]	Fatty acid desaturase 1
0.11[a]	Cytochrome P450, family 3, subfamily a, polypeptide 11
0.23[a]	Aldo-keto reductase family 1, member C12
0.24[a]	ATP-binding cassette, subfamily C (CFTR/MRP), member 2
0.28[a]	Cytochrome P450, family 3, subfamily a, polypeptide 18
0.28[a]	Solute carrier organic anion transporter family, member 1b2
0.28[a]	Complement component 6
0.29[a]	Hydroxysteroid (17-ß) dehydrogenase 2

[a]P<0.05 vs SHRSP

syndrome and that rats may be a useful tool to investigate the molecular mechanisms underlying the metabolic syndrome (11).

In this study, we identified small lipoprotein particles using HPLC. The size of this lipoprotein was similar to the sdLDL, which is one of the major features of lipid abnormality in metabolic syndrome. The existence of apolipoprotein B in this lipoprotein fraction was confirmed by Western blot analysis. From these data, a novel rat model of metabolic syndrome, SHRSP fatty, was described which carries sdLDL like particles, a major characteristic of this disease. Several studies reported differences in LDL particle size, density, and composition between patients with coronary heart disease (CHD) and healthy controls. Prospective, case-control studies confirmed that the presence of sdLDL particles is associated with more than a 3-fold increase in CHD risk (24,25). However, the sdLDL phenotype rarely occurs as an isolated disorder. Instead, it is most frequently accompanied by hypertriglyceridemia, reduced HDL-cholesterol levels, abdominal obesity, and insulin resistance (26,27).

Therefore, the establishment of an animal model carrying sdLDL particles is an important step in examining the mechanisms which underlie the formation of these abnormal lipoproteins. Qiu *et al* reported that hepatic lipase deficient mice have sdLDLs but triglyceride enrichment was not observed in these mice (28), therefore, this is an animal model carrying sdLDLs, but this rat is a model of monogenic disease characterized by hepatic lipase deficiency. In contrast, SHRSP fatty rats have sdLDL-like particles with all of the metabolic syndrome characteristics, and is a useful tool in exploring the mechanism of the formation of lipid abnormalities in metabolic syndrome.

Cholesterol content in HDL fraction separated by gel filtration was significantly higher in SHRSP fatty compared with that in SHRSP. Plasma HDL level of SHRSP fatty is determined by the interaction of carrying genes from leptin receptor region of ZF strain and background SHRSP genes.

Fenofibrate treatment selectively decreased the small particle size of apolipoprotein B containing lipoprotein in SHRSP fatty rats. In the past, fenofibrate was reported to have an effect of increasing LDL particle size and reducing the prevalence of sdLDL in patients (29). Based on these data, the small LDL particles in SHRSP fatty rats have several similarities with human sdLDLs. Therefore, the mechanism of the formation of small particle LDLs in SHRSP fatty seems to overlap that of sdLDLs in humans.

Because of these similarities between the small LDLs in SHRSP fatty rats and human sdLDLs, we conducted an examination of the gene expression resulting in conditions that could be very informative for exploring the underlying mechanism associated with this condition. As the liver is the most important organ in the production of lipid or fatty acid-related metabolism enzymes, we examined liver gene expression in SHRSP fatty rats and control lean SHRSP rats.

Pathologic findings of the liver in the SHRSP fatty rats showed a marked accumulation of lipid droplets in hepatocytes, indicating severe fatty livers. As Adiels *et al* reported, the overproduction of VLDL is driven by the liver fat mass (20). Thus, the examination of the hepatic gene expression profile in SHRSP fatty rats is likely to be very important for understanding the mechanism of this lipid abnormality.

Seventeen genes were upregulated more than 3-fold in the liver tissue of SHRSP fatty rats. Of these genes, at least four including stearoyl coenzyme A desaturase 1 (SCD-1), fatty acid synthase (FAS), ATP citrate lyase, and sterol regulatory element binding factor 1 (SREBF1) are involved in fatty acid metabolism. In fact, SCD-1 gene expression was more than 25 times higher in the liver of SHRSP fatty rats than in control SHRSP rats. Feeding previously fasted animals a low-fat/high-carbohydrate diet caused a marked induction of enzymes involved in catalyzing fatty acid desaturation steps (30), including ATP citrate lyase (31), FAS for lipogenesis (32), and SCD-1. Specifically, SCD-1 catalyzes the introduction of a

double bond in the Δ9 position, between carbons 9 and 10, of a variety of fatty acyl CoA substrates (33), and SCD-1 is also known to play a major role in regulating the fatty acid composition of tissues (34). Ntambi *et al* reported that SCD-/- mice are resistant to diet induced obesity (35). Based on data from animal models, SCD-1 recently became a target of interest for the reversal of hepatic steatosis and insulin resistance (32). Because FAS catalyzes the last step in the fatty acid biosynthetic pathway, it is believed to be a determinant of the maximal capacity of a tissue, including the liver, to synthesize fatty acids via *de novo* lipogenesis. Furthermore, SREBF1 is known to activate the transcription of SCD-1 and FAS, as well as acyl CoA carboxylase-1 in addition to the rate-limiting enzyme in glycerolipid formation, glycerol-3-phosphate acyl-transferase. Therefore, as four of the upregulated genes in the SHRSP fatty rats, SCD-1, FAS, ATP citrate lyase and SREBF play a central role in fatty acid synthesis, upregulation of these genes resulted in increased fat content in the liver and induced overproduction of VLDL particle.

Additionally, our studies also show that some genes were significantly downregulated in SHRSP rats. For example, hepatic lipoprotein overproduction was shown as one of the features of familial combined hyperlipidemia (36), another metabolic disease producing sdLDL particles. Overproduction of these four genes may influence the production of small apolipoprotein B containing particles in SHRSP fatty rats. In contrast, fatty acid desaturase 1 (FADS1) expression was significantly decreased in SHRSP fatty rats compared to SHRSP rats. FADS1 and the human Δ5 desaturase share identical nucleotide sequences in their open reading frame with the exception of 6 alterations. Montanaro *et al* reported that SCD-1 gene expression in the liver of diabetic rats was upregulated, whereas Δ5 desaturase was not significantly modified (37). The downregulated FADS1 and other upregulated genes in our study play distinct roles in liver fatty acid metabolism. As SHRSP fatty rats showed marked accumulation of lipid droplets in hepatocytes, the genes listed in our microarray result analysis play a role in fatty liver.

In conclusion, SHRSP fatty rats demonstrate a novel model of metabolic syndrome, carrying sdLDL like lipoprotein, and thus they may be a good tool for the study of metabolic syndrome. Specifically, some of the upregulated genes in the liver of SHRSP fatty rats, SCD-1, FAS and SREBF, are believed to play a role in the metabolic abnormality of this rat model, and these genes are likely to be a therapeutic target of this metabolic syndrome.

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