Original Article

Effect of Cerivastatin on Endothelial Dysfunction and Aortic CD36 Expression in Diabetic Hyperlipidemic Rats

Tetsuya NAKAMURA, Yuichiro SAITO *, Yoshio OHYAMA *, Tsuyoshi UCHIYAMA *,
Hiroyuki SUMINO *, and Masahiko KURABAYASHI *

A mutation of the CD36 gene that encodes a fatty acid transporter has been reported to play a role in insulin resistance in spontaneously hypertensive rat (SHR). Statins reduce circulating cholesterol and triglyceride concentrations. The objective of this study was to determine the role of CD36 and the significance of statin therapy in insulin-resistance syndromes. We determined the isometric relaxation induced by acetylcholine or lecithinized superoxide dismutase (SOD) in aortas obtained from Otsuka Long Evans Tokushima Fatty (OLETF) rats, a model of insulin resistance and dyslipidemia, and normal control (Long Evans Tokushima Otsuka; LETO) rats with or without cerivastatin treatment. We also determined the effect of cerivastatin on aortic expression of CD36 and PPAR. The CD36 genotype and microsatellite markers on chromosome 4 were also determined. The relaxation induced by acetylcholine and lecithinized SOD were attenuated in OLETF rats but restored by a low dose of cerivastatin without significant changes in serum cholesterol. These relaxations were also restored by a high dose of cerivastatin with significant reductions in serum cholesterol and triglyceride. Cerivastatin increased the aortic expression of CD36 and PPAR mRNA in both LETO and OLETF rats. However, the basal level of CD36 mRNA and the increase in CD36 mRNA in response to cerivastatin were significantly lower in OLETF rats than in LETO rats. Although the abnormal CD36 genotype reported in SHR was not found in OLETF rats, the microsatellite markers of D4Rat151 and D4Rat115 differed between OLETF and LETO rats. In conclusion, insulin resistance in OLETF rats may be partially due to an altered expression of CD36. Increased aortic expression of CD36 in response to cerivastatin could explain the reduction in serum triglyceride concentrations with statin therapy and may have pronounced beneficial effects in insulin-resistance syndromes. (*Hypertens Res* 2004; 27: 589–598)

Key Words: endothelium, superoxide dismutase, insulin

Introduction

Insulin resistance and dyslipidemia are important cardiovascular risk factors that often coexist in patients with type II diabetes, essential hypertension, or other clinical disorders, even in the absence of obesity (1–3). However, the mechanisms responsible for this clustering of metabolic risk factors for cardiovascular disease remain poorly understood. Al-

though primary abnormalities in insulin sensitivity can give rise to disorders of lipid metabolism, a considerable body of evidence suggests that primary defects in lipid metabolism may also cause insulin resistance and impaired glucose tolerance. In the most widely studied rat model of spontaneous hypertension, a naturally occurring mutation in the gene for CD36 that encodes a fatty acid transporter has been identified as a primary determinant of both insulin resistance and dyslipidemia (4, 5). Therefore, at least in the rat, sponta-

From the Clinical Investigation and Research Unit and * Department of Cardiovascular Medicine, Gunma University Hospital, Maebashi, Japan.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant 14570637).

Address for Reprints: Tetsuya Nakamura, M.D., Clinical Investigation and Research Unit, Gunma University School of Medicine, 3–39–22 Showa-machi, Maebashi 371–8511, Japan. E-mail: nakamurt@showa.gunma-u.ac.jp

Received December 24, 2003; Accepted in revised form April 14, 2004.

neous variations in a gene that regulates lipid metabolism can contribute to the inherited variation in insulin action.

The Otsuka Long Evans Tokushima Fatty (OLETF) strain of rat is a useful model of obese type II diabetes characterized by hypertension, hypertriglyceridemia, hyperinsulinemia, hyperglycemia, insulin resistance, and abundant visceral fat (6–8). Their lean littermates are Long Evans Tokushima Otsuka (LETO) rats. Genome-wide quantitative trait locus (QTL) analyses have identified 12 statistically significant QTLs that are associated with the type II diabetes, obesity, or dyslipidemia phenotypes of the OLETF rat (6–8). Using genetic dissection with two kinds of F2 intercross progeny generated by mating between the OLETF rat and non-diabetic F344 or Brown Norway (BN) rats, QTLs associated with features of diabetes have been found on chromosomes 1, 7 and 14, designated as diabetes mellitus, OLETF type Dmo1, Dmo2, and Dmo3, respectively (6–8). Significant loci on chromosomes 4 and 13 have also been identified in the [OLETF × BN] F2 cross (6–8). These results indicate that diabetes in the OLETF rat is polygenic, and that different phenotypic effects are produced by different genetic backgrounds from the same locus.

The spontaneously hypertensive rat (SHR) is another model of hypertension and insulin-resistance syndromes (4, 9). The QTLs for SHR defects in glucose and fatty acid metabolism, hypertriglyceridemia, and hypertension map to a single region on rat chromosome 4 (10). Aitman *et al.* (4) have identified a deletion in the SHR gene for CD36 that encodes a fatty acid receptor/transporter involved in the transmembrane transport of long-chain fatty acids in adipose tissue and in cardiac and skeletal muscle. Genetic analysis of SHR derived from a National Institute of Health colony led to the identification of a causative mutation in the SHR CD36 on chromosome 4 (4). The findings in human CD36 deficiency bear strong resemblance to the phenotype of increased plasma triglycerides and fatty acid concentrations and insulin resistance in rodent CD36 deficiency (11). In the face of such an atherogenic milieu, rodents with CD36 deficiency might be expected to show endothelial dysfunction and premature, accelerated atherosclerosis. In fact, transgenic rescue of defective CD36 ameliorates insulin resistance in SHR (12).

The clinical benefits of cholesterol reduction have been established in large-scale primary and secondary intervention trials using statins, demonstrating that treatment with these drugs results in decreased morbidity and mortality due to coronary heart disease (13). Statin exerts pleiotropic properties and interferes with various vascular events leading to the formation of atherosclerotic lesions by acting on smooth muscle cells, endothelial cells, and macrophages (14–16). Statins increase endothelial nitric oxide synthase expression in cultured human endothelial cells (17). Furthermore, powerful 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, such as simvastatin or atorvastatin, not only reduce circulating cholesterol concentrations but also

reduce triglyceride concentrations (18), in which CD36 may also have a role through its action of fatty acid transport.

In the present study, we investigated the effect of the HMG-CoA reductase inhibitor cerivastatin on the endothelial function and the regulation of expression of CD36 in diabetic hyperlipidemic rats. We also investigated the CD36 genotype in OLETF rats to elucidate the genetic mechanisms of insulin resistance in obesity-induced hypertension.

Methods

Animal Preparation

All procedures were performed in accordance with our institutional guidelines for animal research. Male OLETF rats and LETO rats (6 weeks old) were a gift from Otsuka Pharmaceuticals (Tokushima, Japan). Thirty-week-old male OLETF and LETO rats were fed standard laboratory chow (Oriental Koubo, Tokyo, Japan) that contained 5.5 mEq of sodium and 12.2 mEq of potassium per 100 g of chow, and given distilled water *ad libitum*. Groups of rats received low-dose (0.002 mg/l in the drinking water) or high-dose (0.006 mg/l in the drinking water) cerivastatin or distilled water for 4 weeks using the following protocols:

1) LETO vehicle group ($n = 10$): Distilled water was provided to LETO rats for 4 weeks. 2) LETO cerivastatin 0.002 mg/ml group ($n = 9$): Cerivastatin (0.002 mg/ml) was administered to LETO rats in the drinking water for 4 weeks. In preliminary studies, we determined that the presence of cerivastatin in the drinking water did not affect the rats' drinking behavior. The daily intake of cerivastatin was approximately 0.2 mg/kg. 3) LETO cerivastatin 0.006 mg/ml group ($n = 12$): Cerivastatin (0.006 mg/ml) was administered to LETO rats in the drinking water for 4 weeks. The daily intake of cerivastatin was approximately 0.6 mg/kg. 4) OLETF vehicle group ($n = 8$): Distilled water was provided to OLETF rats for 4 weeks. 5) OLETF cerivastatin 0.002 mg/ml group ($n = 10$): Cerivastatin (0.002 mg/ml) was administered to OLETF rats in the drinking water for 4 weeks. 6) OLETF cerivastatin 0.006 mg/ml group ($n = 13$): Cerivastatin (0.006 mg/ml) was administered to OLETF rats in the drinking water for 4 weeks.

The systolic blood pressure was measured by the tail-cuff method in each group (UR-5000; Ueda Co., Tokyo, Japan). Four weeks after starting cerivastatin, the rats were anesthetized with pentobarbital (40 mg/kg i.p.). Serum concentrations of creatinine, sodium, potassium, glucose, total cholesterol and triglyceride were determined.

Effect of Cerivastatin on Endothelium-Dependent Vascular Responses in OLETF Rats

Aortic ring preparations were obtained from OLETF and LETO rats with or without cerivastatin treatment. Cylindrical segments 3.0-mm long were cut from the aorta and were

Table 1. Primers Used

Genotype		Primer
CD36	Foward	TCAAGGTGTGCTCAACAGCC
	Reverse	AGGATAAAACACACCAACTGT
PPAR γ	Foward	ATAAAGTCCTTCCCGCTGACCAAAGCC
	Reverse	GCGGTCTCCACTGAGAATAATGACAGC
D4Rat9	Foward	TTTATGGTTTAATTTGGTGTCTTTG
	Reverse	CCTTGGAAGGTTTGGACCA
D4Rat151	Foward	GGTGCAAATTCAGAGGCATT
	Reverse	TCAAAGGGTAGATGATGGAGTTT
D4Rat115	Foward	CCAGCTCACGAACACACATT
	Reverse	CCGTGGTTTAGATACTATGCCTTC
D4Rat101	Foward	TGGATGAAGTCCTGTAATGCA
	Reverse	TGGTCATGACTATCCAGTGACA
D4Rat24	Foward	AGGGACACTGATGTAGCCATG
	Reverse	GATAAAATCTATGCTTAAGCCCC

PPAR γ , peroxisome proliferator-activated receptor γ .

bathed in 10 ml of Krebs bicarbonate saline (120 mmol/l NaCl; 5.2 mmol/l KCl; 2.4 mmol/l CaCl₂; 1.2 mmol/l MgSO₄; 25 mmol/l NaHCO₃; 0.03 mmol/l Na₂-EDTA; and 11 mmol/l dextrose [pH, 7.4]) equilibrated with 95% O₂ and 5% CO₂, and maintained at 37°C.

The rings were suspended under 2 g of tension and pre-constricted by adding 10⁻⁷ mol/l norepinephrine. The force of isometric contraction was measured using a force-displacement transducer (Model UR-50GR; Minebea Co., Nagano, Japan). After the contraction force had reached a plateau, acetylcholine 10⁻⁸ to 10⁻⁵ mol/l was added incrementally to the bath. The effects of NO synthesis inhibition with N^G-nitro-L-arginine methyl ester (LNAME, 10⁻⁵ mol/l) were also studied. To confirm that the vascular smooth muscle cells were intact, the vasodilator response to sodium nitroprusside (10⁻¹⁰ to 10⁻⁷ mol/l) was determined. The amount of superoxide necessary to shorten the half-life of endothelium-derived NO was also evaluated by administering lecithinized superoxide dismutase (lecithinized SOD) (0.25 to 4.0 units/l) (19, 20). Acetylcholine (Sigma Chemical Co., St. Louis, USA) and LNAME (Calbiochem Co., La Jolla, USA) were dissolved in Krebs bicarbonate solution.

In each experimental group, *n* refers to the number of animals from which the aortic rings were obtained. The maximum vasodilator response was defined as the level of vasodilation that preceded the precontraction induced by norepinephrine. The response to each dose of drug is expressed as a percent of the maximum vasodilation as described previously (19, 20).

Measurements of CD36 and Peroxisome Proliferator-Activated Receptor (PPAR γ) mRNA Expression in the Aorta

RNA Isolation from Thoracic Aortas

The thoracic aorta, extending from the aortic root to the second intercostal region and up to the carotids, was weighed, flash immersed in RNA stabilization reagent (RNAlater; QIAGEN Inc., Valencia, USA) and stored at -80°C. Isolation of total RNA was performed using an RNeasy kit (QIAGEN Inc.). Total RNA was treated with deoxyribonuclease I (QIAGEN Inc.) for 20 min at room temperature to remove contaminating genomic DNA. The amount of RNA was determined by spectrophotometry, and 200 ng of RNA was loaded onto a 1.5% agarose gel to determine its quality before analysis.

Reverse-Transcription (RT)-Polymerase Chain Reaction (PCR)-Based Quantitative Gene Expression Analysis

Real-time detection of PCR products was performed. Using the Perkin-Elmer ABI Prism 7700 and Sequence Detection System software (Perkin-Elmer, Foster City, USA), the differential displays of mRNAs for CD36 and PPAR γ were determined. Briefly, 1 μ g of total RNA was used to generate cDNA using an oligo dT oligodeoxynucleotide primer (T12-18) following the protocol for Omniscript (QIAGEN Inc.). The primers for CD36 and PPAR γ are shown in Table 1, and the following probes were used: CD36: 5'-GAAGGA ACTCTTGTGGGGTTAC-3'; PPAR γ : 5'-CCTGCGGAAG CCCTTTGGTGACT-3'. The TaqMan Rodent GAPDH Control Reagents (Perkin-Elmer) were used to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. Equal amounts of cDNA were used in triplicate and amplified with the TaqMan Master Mix provided by Perkin-Elmer. Amplification efficiencies were validated and normalized against

Table 2. Effects of Cerivastatin on Body Weight, Systolic BP, Serum Glucose, Triglyceride and Total Cholesterol in LETO and OLETF Rats

	LETO rats			OLETF rats		
	Distilled water (n = 10)	Low dose (n = 9)	High dose (n = 12)	Distilled water (n = 8)	Low dose (n = 10)	High dose (n = 13)
Body weight (g)	504 ± 10	520 ± 10	523 ± 8	628 ± 15*	615 ± 16 [#]	581 ± 12 [¶]
Systolic BP (mmHg)	134 ± 4	140 ± 3	144 ± 3	151 ± 3*	163 ± 4 [#]	158 ± 3 [¶]
Serum glucose (mg/dl)	166 ± 9	154 ± 6	158 ± 10	236 ± 17*	225 ± 12 [#]	203 ± 13 [¶]
Triglyceride (mg/dl)	54 ± 8	69 ± 6	60 ± 5	165 ± 17*	155 ± 16 [#]	112 ± 13 ^{¶, †}
Total cholesterol (mg/dl)	93 ± 2	96 ± 2	92 ± 2	115 ± 3*	109 ± 4 [#]	94 ± 2 [†]

Data are mean ± SEM. BP, blood pressure; LETO, Long Evans Tokushima Otsuka; OLETF, Otsuka Long Evans Tokushima Fatty; Low dose, cerivastatin 0.002 mg/ml in drinking water; High dose, cerivastatin 0.006 mg/ml in drinking water. **p* < 0.05 vs. LETO rats, distilled water. [#] *p* < 0.05 vs. LETO rats, low dose. [¶] *p* < 0.05 vs. LETO rats, high dose, [†] *p* < 0.05 vs. OLETF rats, distilled water.

Table 3. Effects of Cerivastatin on Serum Creatinine, BUN, Electrolytes, AST and ALT in LETO and OLETF Rats

	LETO rats			OLETF rats		
	Distilled water (n = 10)	Low dose (n = 9)	High dose (n = 12)	Distilled water (n = 8)	Low dose (n = 10)	High dose (n = 13)
Serum creatinine (mg/dl)	0.43 ± 0.01	0.52 ± 0.04	0.46 ± 0.02	0.32 ± 0.01	0.36 ± 0.01	0.35 ± 0.01
Serum BUN (mg/dl)	24.4 ± 0.8	23.6 ± 0.5	23.1 ± 0.5	22.2 ± 1.0	21.8 ± 0.3	21.3 ± 0.5
Serum sodium (mEq/l)	144.3 ± 0.7	140.9 ± 0.5	142.0 ± 0.5	142.3 ± 0.6	141.0 ± 0.4	141.9 ± 0.6
Serum potassium (mEq/l)	5.1 ± 0.1	5.2 ± 0.3	4.7 ± 0.1	5.0 ± 0.1	5.0 ± 0.2	4.8 ± 0.2
AST (IU/l)	131 ± 7	159 ± 11	149 ± 7	120 ± 5	108 ± 8	129 ± 10
ALT (IU/l)	50 ± 2	43 ± 1	44 ± 2	57 ± 5	46 ± 2	54 ± 6

Data are mean ± SEM. Low dose, cerivastatin 0.002 mg/ml in drinking water; High dose, cerivastatin 0.006 mg/ml in drinking water; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LETO, Long Evans Tokushima Otsuka; OLETF, Otsuka Long Evans Tokushima Fatty.

GAPDH, and nanograms of product were calculated using the standard curve method for quantitation against cDNA that was reverse-transcribed from isolated aortas of OLETF and LETO rats. Total RNA that was not reverse-transcribed was also analyzed to determine genomic DNA contamination.

CD36 Genotyping

We analyzed the CD36 genotypes in both OLETF (*n* = 80) and LETO rats (*n* = 80) using PCR and a high throughput capillary sequencer (ABI PRISM 3100 Genetic Analyzer; Perkin-Elmer). PCR amplification of exon 6 of CD36 between 486 and 633 bp (Genbank accession no. L19658) was performed using the same primers as for the real-time RT-PCR (Table 1). PCR products were purified with a PCR purification kit (Perkin Elmer) and directly sequenced using a Big Dye sequencing kit (Perkin Elmer) on an ABI Prism 3100 Genetic Analyzer. This sequencing detects both the normally transcribed wild-type copy of CD36 and the SHR deletion variant in CD36 described previously by Aitman *et al.* (4).

Microsatellite Genotyping

The size of the differential segment was estimated by genotyping of microsatellite markers on chromosome 4 in both OLETF (*n* = 20) and LETO rats (*n* = 20) (21). Primer sets for microsatellite markers of chromosome 4 (D4Rat9, D4Rat151, D4Rat115, D4Rat101, and D4Rat24) were obtained from commercial sources (MapPairs; ResGen Inc., Invitrogen, Boston, USA) and are shown in Table 1. The forward and reverse primers were 5' fluorescent-labeled with FAM. PCR was performed according to the manufacturer's protocol. PCR products were visualized on an ABI Prism 3100 Genetic Analyzer.

Statistical Analyses.

All data are expressed as the mean ± SEM. Aortic vasodilation, biochemical markers, blood pressure, body weights and aortic expressions of CD36 and PPAR γ mRNA during the course of the study were analyzed by ANOVA. Statistical significance was defined as *p* < 0.05.

Fig. 1. Vasodilation induced by acetylcholine, lecithinized superoxide dismutase (SOD) and sodium nitroprusside in LETO or OLETF rats treated with or without cerivastatin. Open and closed circles represent rats receiving no drug. Open and closed squares represent rats treated with cerivastatin 0.002 mg/ml. Open and closed triangles represent rats treated with cerivastatin 0.006 mg/ml. Open circles, squares, and triangles represent experiments without N^G -nitro-L-arginine methyl ester (LNAME) 10^{-5} mol/l in the tissue bath. Closed circles, squares, and triangles represent experiments with LNAME in the tissue bath. The vasodilator response to each dose of drug is expressed as a percent of the level that preceded the precontraction induced by norepinephrine. * $p < 0.05$ compared with rats given distilled water without LNAME.

Results

The systolic blood pressure and body weight were significantly higher in OLETF rats under the treatments with distilled water, low-dose cerivastatin, or high-dose cerivastatin for 4 weeks than in LETO rats under the corresponding treatments, respectively (Table 2). Serum concentrations of glucose and triglycerides were significantly higher in OLETF rats compared with LETO rats. The treatment with high doses of cerivastatin (0.006 mg/ml in the drinking water) de-

creased serum concentrations of cholesterol and triglycerides significantly only in OLETF rats. The treatment with low-dose cerivastatin (0.002 mg/ml in the drinking water) did not affect concentrations of cholesterol or triglycerides significantly in either OLETF or LETO rats (Table 2). There were no significant differences in the serum creatinine, urea nitrogen, sodium, or potassium concentrations or the aspartate aminotransferase or alanine aminotransferase activities among the groups (Table 3).

The relaxation induced by acetylcholine and lecithinized SOD was attenuated in OLETF compared with LETO rats

Fig. 2. Real-time PCR analysis of aortic expression of CD36 and PPAR γ . Open circles represent rats treated with cerivastatin 0.006 mg/ml. Closed squares represent rats receiving no drug. Horizontal bold lines represent the threshold level. ΔRn represents the normalized reporter signal minus the baseline signal established in the first few cycles of PCR. ΔRn increases during PCR as the target is amplified, until the reaction approaches a plateau.

Fig. 3. Effect of cerivastatin on the aortic expression of CD36 mRNA and PPAR γ mRNA in LETO and OLETF rats. Rats were given distilled water (Vehicle), cerivastatin (0.002 mg/ml), or cerivastatin (0.006 mg/ml). Open and closed bars represent LETO and OLETF rats, respectively. N = 8 in each group. Data are reported as the mean $\pm$ SEM. * $p < 0.05$ compared with rats given distilled water (Vehicle). † $p < 0.05$ compared with LETO rats.

and was abolished by the NO synthase inhibitor, LNAME (Fig. 1). Treatment with low-dose cerivastatin restored the relaxation induced by acetylcholine and lecithinized SOD in OLETF rats without significantly affecting the serum cholesterol or triglyceride concentrations. Treatment with high-dose cerivastatin significantly reduced the serum cholesterol and triglyceride concentrations (Table 2) and also restored the relaxation by acetylcholine and lecithinized SOD in

OLETF (Fig. 1).

The levels of CD36 mRNA expression in the aorta of OLETF rats were significantly lower than in LETO rats (Figs. 2 and 3). Treatment with low- and high-dose cerivastatin significantly increased the expression of CD36 mRNA in both LETO and OLETF rats. Although treatment with low-dose cerivastatin did not change PPAR γ mRNA significantly in either LETO or OLETF rats, treatment with high-

Fig. 4. CD36 genotypes in both OLETF ($n = 80$) and LETO rats ($n = 80$). Shown are DNA sequence and electropherograms for CD36 in LETO and OLETF rats. Sequencing with forward (upper) and reverse (lower) primers gave the same results.

Fig. 5. Analyses of the microsatellite markers of D4Rat151 and D4Rat115 in OLETF. Shown are the GeneScan histograms showing the PCR fragment lengths (base pairs; x-axis) for the blood specimens plotted against the PCR fragment peak height (y-axis) after a high-resolution fluorescent electrophoresis run. The peak height is directly proportional to the number of fluorescent fragments detected by the ABI 3100 Genetic Analyzer after capillary electrophoresis. Note the different migration patterns of the PCR fragment peaks for D4Rat151 and D4Rat115 between OLETF and LETO rats in comparison to the molecular weight marker.

dose cerivastatin increased the expression of PPAR γ mRNA in both LETO and OLETF rats (Figs. 2 and 3). The genotypes of CD36, determined by direct sequencing, were identical between OLETF and LETO rats ($n = 80$ /group) (Fig. 4). The microsatellite markers of D4Rat9, D4Rat101, and D4Rat24 in OLETF were identical with those in LETO rats. However, the microsatellite markers D4Rat151 and D4Rat115 differed between OLETF and LETO rats ($n = 20$ /group) (Fig. 5).

Discussion

We tested for the presence of hyperglycemia, dyslipidemia, high blood pressure, and endothelial dysfunction in 34-week-old male OLETF and LETO rats. We have shown that endothelial dysfunction, metabolic disturbances, and hypertension are present in OLETF rats. In previous linkage studies in the OLETF rat, putative QTLs for increased blood pressure and abnormal carbohydrate and lipid metabolism were mapped to relatively broad regions of chromosome 4. Kanemoto *et al.* (6) targeted their genetic dissection studies on male OLETF progenies of [female OLETF $\times$ male control rats] F2 crosses, and the population included 164 male [OLETF $\times$ BN] F2 rats. They detected QTLs on chromosome 4 in the [OLETF $\times$ BN] F2 cross, which showed linkage to serum glucose concentrations at 60 min (lod = 3.02) and at 90 min (lod = 3.00) of an oral glucose tolerance test to markers D4Wox10 and D4Mgh7, respectively. The locus of CD36 is located at approximately 30 cM away from D4Wox10 (Fig. 6). Gauguier and colleagues (22) also mapped a QTL linked to postprandial insulin secretion in the region of chromosome 4 of Goto-Kakizaki type 2 diabetic rats, which is close to the CD36 locus.

Aitman *et al.* (4) showed that Southern analysis of SHR genomic DNA detected missing bands caused by deletion of

Fig. 6. Linkage map of chromosome 4. Map distances are adapted from chromosome 4 maps of the WIBR/MIT Rat Genome Center (21). The position of CD36 is based on the radiation hybrid mapping results of Aitman *et al.* (4). The asterisk denotes the markers that differ between OLETF and LETO rats.

the normally transcribed copy of CD36. To investigate whether CD36 is responsible for the phenotypic effects linked to D4Wox10, we sequenced the region that distinguishes the deletion variant of CD36 described by Aitman *et al.* (4), but found no differences between OLETF and LETO rats. Though we cannot exclude the possibility of alterations in a distant enhancer region, this result would imply the participation of another gene in this region of rat chromosome 4. In fact, we detected differences in the microsatellite markers of D4Rat151 and D4Rat115 in chromosome 4 between OLETF and LETO rats (Figs. 5 and 6). We know of no other candidate genes mapped to this region, mainly because far fewer genes have been mapped on the rat genome in contrast to the human and mouse.

The findings by Aitman *et al.* (4) raise fundamental questions about the role of CD36 in metabolic and cardiovascular disease and about the relationship between CD36 and the nuclear receptor PPAR γ , the target of the insulin-sensitizing thiazolidinone class of drugs (23). Because PPAR γ transcriptionally induces the CD36 gene, the effects are hard to predict (24, 25). Some of the reported effects of PPAR γ are pro-atherogenic, for example, induction of foam-cell formation from macrophages (24, 26), whereas others, including repression of inflammatory cytokines and induction of cholesterol efflux from macrophages, are potentially anti-atherogenic (25, 27). Miyaoka and colleagues (11) found that their

study group of 26 people with CD36 deficiency had higher plasma triglycerides and glucose concentrations, lower plasma high-density lipoprotein (HDL)-cholesterol concentrations, and much higher blood pressure than did controls. The data showed a striking similarity between the phenotype of these CD36-deficient subjects and the range of metabolic and cardiovascular abnormalities found in the common syndromes of insulin resistance. The findings in human CD36 deficiency also bear a strong resemblance to the phenotypes of increased plasma triglyceride and fatty acid concentrations and insulin resistance in SHR with CD36 deficiency. In the face of such an atherogenic milieu, SHR with CD36 deficiency might be expected to show premature, accelerated atherosclerosis. However, mice with CD36 deficiency have a six-fold reduction in the amount of atherosclerotic plaques compared with control animals (28). The assumption is that the mice with CD36 deficiency are protected from the development of arterial disease because the absence of CD36 results in a failure of uptake of oxidized low-density lipoprotein (LDL) cholesterol by macrophages, reduced foam-cell formation, and a reduced severity of atherosclerotic lesions (28). Whether this presumed mechanism is correct and whether it occurs in human beings must await the results of further studies. However, in the present study, the increase in CD36 expression in response to cerivastatin was accompanied by the restoration of endothelial function in OLETF rats. In the present study, the oral administration of cerivastatin improved aortic endothelial function in OLETF rats; however, blood pressure was not significantly altered by the treatment with cerivastatin. This result implies that microvascular endothelial function, which may have a more major role in blood pressure regulation (29), was not improved by this 4-week treatment with cerivastatin enough to lower blood pressure in OLETF rats.

Fajas *et al.* (30) have shown that PPAR γ protein expression is induced in cells treated with HMG-CoA reductase inhibitors and is not affected by fatty acids. Treatment with HMG-CoA reductase inhibitors, which block the enzyme responsible for the rate-limiting step of cholesterol synthesis, modifies cellular cholesterol levels (30). Cholesterol depletion induced by statins triggers the cleavage of cholesterol-sensitive transcription factors, called SREBPs. Upon treatment with compounds such as mevastatin or simvastatin, cells become cholesterol-depleted and the production of the active forms of ADD-1/SREBP-1 increases (30). Upregulation of ADD-1/SREBP-1 leads to the production of endogenous ligands for PPAR γ (30, 31). Treatment of cells with simvastatin for 6 h results in robust and fast induction of PPAR γ protein levels, which may be sustained 12 h after addition, further supporting the notion that cellular cholesterol concentrations influence the expression of PPAR γ (30, 31).

The regulation of the expression of PPAR γ , a nuclear receptor that is activated by fatty acid metabolites, by the cholesterol-regulated transcription factors of the ADD-1/SREBP-1 family, links transcriptional control by these two

important classes of lipids (30). Changes in intracellular cholesterol levels will, *via* modulation of ADD-1/SREBP-1 and/or SREBP-2 activity, profoundly affect fatty acid and triglyceride metabolism, which is controlled by PPAR γ activity. One interesting example of such an interrelationship between cholesterol and fatty acid metabolism, is the observation that powerful HMG-CoA reductase inhibitors, such as simvastatin or atorvastatin, not only reduce circulating cholesterol but also triglyceride concentrations (18). The triglyceride-lowering effects of cerivastatin were also observed in the present study. If statins cause an induction of PPAR γ expression, as shown by Fajas *et al.* (30) and also by the present study, the increased PPAR γ transcriptional activity would induce the expression of several genes involved in triglyceride clearance, including the CD36 gene. This new data could provide a basis for both the pronounced beneficial effects of the statins in patients with diabetic hyperlipidemia and for the development of agents which have a broader or more specific ability to regulate different aspects of lipid metabolism.

PPAR γ has been shown to stimulate transcription of the CD36 gene (24, 25). Li *et al.* (25) have shown that the PPAR γ -specific agonists rosiglitazone and GW845 increase expression of the CD36 scavenger receptor in the arterial wall and strongly inhibit the development of atherosclerosis in LDL-cholesterol receptor-deficient male mice. Qi *et al.* (32) reported that CD36 is a key determinant of the metabolic effects of pioglitazone. They showed that administration of pioglitazone was associated with significantly lower circulating concentrations of fatty acid, triglyceride, and insulin in congenic and transgenic SHR than in SHR harboring mutant CD36 (32). Pravenec *et al.* (12) reported that transgenic rescue of defective CD36 ameliorates insulin resistance in SHR. These findings confirm that the increase in CD36 expression by cerivastatin in the present study should also ameliorate insulin resistance.

In conclusion, the present study demonstrated that the mechanisms of insulin resistance of OLETF may be partially due to an altered expression of CD36. Although the abnormal CD36 genotype reported in SHR was not found in OLETF rats, a causative gene may still exist on chromosome 4 because of a reported QTL and differences in the microsatellite markers D4Rat151 and D4Rat115 in the present study. Increased expression of CD36 by cerivastatin could be the mechanism responsible for the reduction in serum triglycerides by HMG-CoA reductase inhibitors. Restoration of endothelial function and increased CD36 expression by statin therapy may have pronounced beneficial effects in insulin-resistance syndromes.

Acknowledgements

The authors are grateful to Yumiko Sato for her excellent technical help.

References

1. Reaven GM: Banting lecture 1988: role of insulin resistance in human disease. *Diabetes* 1988; **37**: 1595–1607.
2. Reaven GM, Lithell H, Landsberg L: Hypertension and associated metabolic abnormalities—the role of insulin resistance and the sympathoadrenal system. *N Engl J Med* 1996; **334**: 374–381.
3. Reaven GM: Importance of identifying the overweight patient who will benefit the most by losing weight. *Ann Intern Med* 2003; **138**: 420–423.
4. Aitman TJ, Glazier AM, Wallace CA, *et al*: Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* 1999; **21**: 76–83.
5. Pravenec M, Zidek V, Shimokova M, *et al*: Genetics of Cd36 and the clustering of multiple cardiovascular risk factors in spontaneous hypertension. *J Clin Invest* 1999; **103**: 1651–1657.
6. Kanemoto N, Hishigaki H, Miyakita A, *et al*: Genetic dissection of “OLETF,” a rat model for non-insulin-dependent diabetes mellitus. *Mammalian Genome* 1998; **9**: 419–425.
7. Watanabe TK, Okuno S, Ono T, *et al*: Single-allele correction of the Dmo1 locus in congenic animals substantially attenuates obesity, dyslipidaemia and diabetes phenotypes of the OLETF rat. *Clin Exp Pharmacol Physiol* 2001; **28**: 28–42.
8. Okuno S, Watanabe TK, Ono T, *et al*: Effects of Dmo1 on obesity, dyslipidaemia and hyperglycemia in the Otuska Long Evans Tokushima Fatty strain. *Genet Res* 2001; **77**: 183–190.
9. Cui ZH, Nemoto K, Kawakami K, Gonda T, Nabika T, Masuda J: Fine linkage mapping of the blood pressure quantitative trait locus region on rat chromosome 1. *Hypertens Res* 2002; **25**: 605–608.
10. Aitman TJ, Gotoda T, Evans AL, *et al*: Quantitative trait loci for cellular defects in glucose and fatty acid metabolism in hypertensive rats. *Nat Genet* 1997; **16**: 197–201.
11. Miyaoka K, Kuwasako T, Hirano K, Nozaki S, Yamashita S, Matsuzawa Y: CD36 deficiency associated with insulin resistance. *Lancet* 2001; **357**: 686–687.
12. Pravenec M, Landa V, Zidek V, *et al*: Transgenic rescue of defective Cd36 ameliorates insulin resistance in spontaneously hypertensive rats. *Nat Genet* 2001; **27**: 156–158.
13. Hebert PR, Gaziano JM, Chan KS, Hennekens CH: Cholesterol lowering with statin drugs, risk of stroke, and total mortality: an overview of randomized trials. *JAMA* 1997; **278**: 313–321.
14. Bellosta S, Ferri N, Arnaboldi L, Bernini F, Paoletti R, Corsini A: Pleiotropic effects of statins in atherosclerosis and diabetes. *Diabetes Care* 2000; **23** (Suppl 2): B72–B78.
15. Vasa M, Fichtlscherer S, Adler K, *et al*: Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* 2001; **103**: 2885–2890.
16. Tsunekawa T, Hayashi T, Kano H, *et al*: Cerivastatin, a hydroxymethylglutaryl coenzyme A reductase inhibitor, improves endothelial function in elderly diabetic patients

- within 3 days. *Circulation* 2001; **104**: 376–379.
17. Lauf U, Liao JK: Post-transcriptional regulation of endothelial nitric oxide synthesis mRNA stability by Rho GTPase. *J Biol Chem* 1998; **273**: 24266–24271.
 18. Alaupovic P, Heinonen T, Shurzinske L, Black DM: Effect of a new HMG-CoA reductase inhibitor, atorvastatin, on lipids, apolipoproteins and lipoprotein particles in patients with elevated serum cholesterol and triglyceride levels. *Atherosclerosis* 1997; **133**: 123–133.
 19. Hoshino J, Sakamaki T, Nakamura T, *et al*: Exaggerated vascular response due to endothelial dysfunction and role of the renin-angiotensin system at early stage of renal hypertension in rats. *Circ Res* 1994; **74**: 130–138.
 20. Nakamura T, Kurashina T, Saito Y, *et al*: Lecithinized superoxide dismutase induces vasodilation; evidence of direct contribution of superoxide anions to modulating vascular tone. *Life Sci* 1999; **64**: PL65–PL70.
 21. Rat Genome Center: <http://www.genome.wi.mit.edu/rat/public/>
 22. Gauguier D, Froguel P, Parent V, *et al*: Chromosomal mapping of genetic loci associated with non-insulin dependent diabetes in the GK rat. *Nat Genet* 1996; **12**: 38–43.
 23. Aitman TJ: CD36, insulin resistance, and coronary heart disease. *Lancet* 2001; **357**: 651–652.
 24. Nagy L, Tontonoz P, Alvarez JGA, Chen H, Evans RM: Oxidized LDL regulates macrophage gene expression through ligand activation of PPAR γ . *Cell* 1998; **93**: 229–240.
 25. Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Glass CK: Peroxisome proliferator-activated receptor γ ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* 2000; **106**: 523–531.
 26. Tontonoz P, Nagy L, Alvarez JGA, Thomazy VA, Evans RM: PPAR γ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 1998; **93**: 241–252.
 27. Chinetti G, Lestavel S, Bocher V, *et al*: PPAR- α and PPAR- γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med* 2001; **7**: 53–58.
 28. Febbraio M, Podrez EA, Smith JD, *et al*: Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J Clin Invest* 2000; **105**: 1049–1056.
 29. Yoshida Y, Ohyanagi M, Iwasaki T: Chronological changes of α -adrenoceptor-mediated vascular constriction in Otsuka-Long-Evans-Tokushima fatty rats. *Hypertens Res* 2003; **26**: 559–567.
 30. Fajas L, Schoonjans K, Gelman L, *et al*: Regulation of peroxisome proliferator-activated receptor γ expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein1: implications for adipocyte differentiation and metabolism. *Mol Cell Biol* 1999; **19**: 5495–5503.
 31. Martin G, Duez H, Blanquart C, *et al*: Statin-induced inhibition of the Rho-signaling pathway activates PPAR α and induces HDL apoA-I. *J Clin Invest* 2001; **107**: 1423–1432.
 32. Qi N, Kazdova L, Zidek V, *et al*: Pharmacogenetic evidence that CD36 is a key determinant of the metabolic effects of pioglitazone. *J Biol Chem* 2002; **277**: 48501–48507.