Congenic BB.SHR Rat Provides Evidence for Effects of a Chromosome 4 Segment (*D4Mit6-Npy* ~1 cm) on Total Serum and Lipoprotein Lipid Concentration and Composition After Feeding a High-Fat, High-Cholesterol Diet

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Congenic BB.SHR (previously referred to as BB.LL) rats were generated by transferring the segment of chromosome 4 flanked by the *D4Mit6* and *Spr* loci from the spontaneously hypertensive rat (SHR/Mol) onto the genetic background of the diabetes-prone BB/OK rat. In this study, the influence of the above-mentioned region of chromosome 4 on triglyceride, cholesterol, and phospholipid phenotypes after a high-fat, high-cholesterol diet was examined by comparison of BB.SHR congenic rats with BB/OK rats. BB/OK and BB.SHR had comparable concentrations of basal and postdietary serum insulin, as well as of basal total serum triglycerides and had an identical body weight and food intake at the beginning of the test period. However, after 4 weeks on the test diet, BB.SHR rats were significantly heavier than BB/OK rats and had significantly higher food intake and lower total serum triglyceride concentrations. The basal serum leptin level was significantly lower, but postdietary serum leptin concentration did not show a significant difference between the 2 strains. Furthermore, significantly higher basal total serum cholesterol and phospholipid levels were observed in BB.SHR rats, but this difference disappeared after feeding the high-fat, high-cholesterol diet. Postdietary high-density lipoprotein $(HDL)_2$ cholesterol and phospholipid levels were significantly elevated in BB.SHR rats when compared with BB/OK rats. The 2 strains also differed slightly, but significantly, with respect to the other HDL phospholipid concentrations. In addition to previously described differences between BB/OK and BB.SHR rats, the results of this study clearly show the impact of genes, lying within the transferred segment, on serum lipid phenotypes after high-fat, high-cholesterol diet.

TO IDENTIFY DISCRETE genetic factors contributing to complex quantitative traits, such as body weight, blood pressure, or lipids, 2 inbred strains markedly differing in the trait of interest are normally crossed to generate F1 hybrids, which are either intercrossed to generate F2 hybrids, or are backcrossed onto 1 of the 2 inbred strains to generate first backcross hybrids (BC1). The phenotypes and genotypes of the F2 and BC1 hybrids are determined and the data are used for linkage analysis to identify quantitative trait loci (QTLs).[1] To study QTLs for blood pressure and blood pressure-related traits, diabetes-prone B(io) B(reeding)/O(ttawa) K(arlsburg) and S(pontaneously) H(ypertensive) R(at)/Mol(legaard) rats were crossed and backcrossed onto BB/OK, and finally, the phenotype and genotype of backross hybrids were analyzed. The data showed a suggestive and significant linkage on chromosome 4 around the *Npy* locus for total serum cholesterol and triglyceride levels, respectively.[2,3] To examine the physiologic impact of the described region, congenic BB.SHR rats were generated by transferring the segment of chromosome 4 flanked by the *D4Mit6* and *Spr* loci from the SHR/Mol onto the genetic background of the BB/OK rat.[4] The phenotypic characterization of congenic BB.SHR rats showed that these rats do not develop diabetes, but are characterized by higher basal total serum triglycerides and cholesterol concentration when compared with BB/OK rats, confirming the described QTLs for lipid phenotypes in the BB/OK × SHR/Mol crossing study.[4] In addition, it was shown that BB.SHR were significantly heavier than the BB/OK rats at an age of 16 weeks.[4]

Recently, using a large set of congenic and recombinant inbred strains derived from SHR/Ola and BN-Lx/Cub, it was suggested that different chromosomal regions (including a region of chromosome 4) were associated with basal (rats on a low-fat, low-cholesterol diet) and postdietary (rats on high-fat, high-cholesterol diet) cholesterol and phospholipid phenotypes.[5-7] The SHR.BN congenic strain used in these studies was generated by transferring a segment of chromosome 4 (*Il6-D4Mit6-Npy*, > 30 cm) from the BN rat onto the SHR background. Despite evident effects of this region on lipid phenotypes in SHR.BN congenics, the region is very large, and it is difficult to determine which genes could be considered to be serious candidates for further genetic analysis. The BB.SHR congenics offer a possibility to show whether the region *D4Mit6-Npy-Spr* (~12 cm), which partially overlaps the region *Il6-D4Mit6-Npy* transferred in SHR.BN congenics (Fig 1), has an impact on lipid postdietary phenotypes. In this case, it would mean that not the genes close to *Il6*, such as *Cd36*, but gene or genes lying within the region *D4Mit6-Npy* (~1 cm) are responsible for genetic control of lipid phenotypes in the rat. To show the influence of the *D4Mit6-Npy-Spr* segment of chromosome 4 in the BB.SHR rat, not only basal, but also postdietary total

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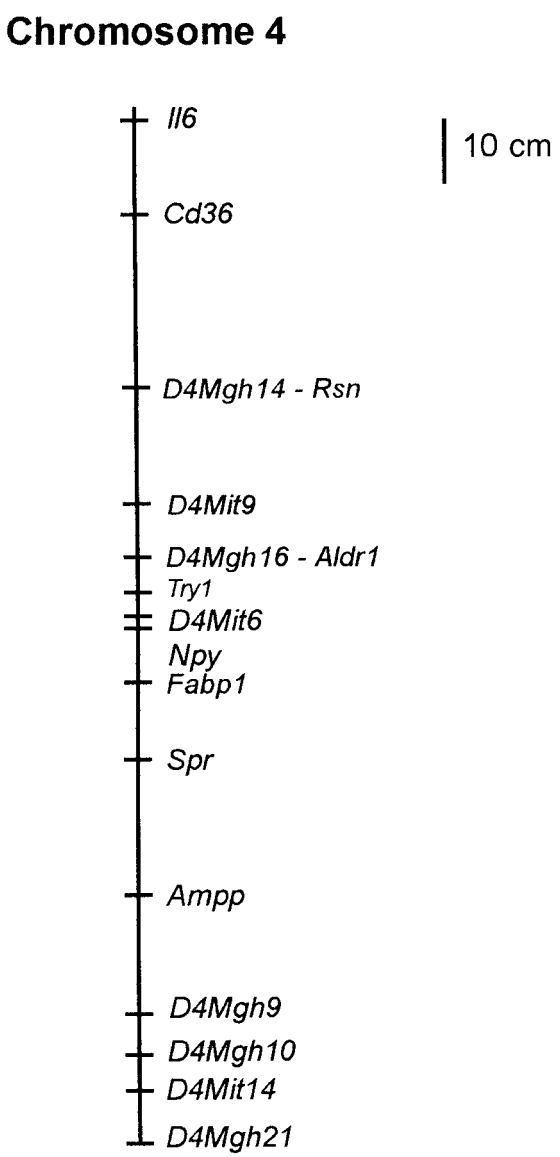

Fig 1. Genetic linkage map of the rat chromosome 4.

serum lipid levels, should be studied. In addition to measuring total serum lipid levels, it is also relevant to measure the concentrations of the lipoprotein subfractions. This prompted the present study in which BB.SHR congenic rats were compared with BB/OK rats for total serum and lipoprotein lipid (cholesterol and phospholipid) concentration and composition after feeding them a high-fat, high-cholesterol diet.

MATERIALS AND METHODS

Experimental Animals

The BB/OK inbred and BB.SHR congenic strain are maintained at the Department of Laboratory Animal Science in Karlsburg (Germany). The congenic strain BB.SHR was generated by cross of BB/OK and SHR/Mol rats, with repeated backcrossing of cross hybrids onto diabetic BB/OK rats. The cross hybrids were selected for heterozygosity of region of chromosome 4 (*D4Mit6-Npy-Spr*) and for homozygosity for BB/OK alleles at 72 background loci examined. After 5 backcross generations, the animals were already homozygous at background loci and were intercrossed. Animals homozygous for SHR alleles at the loci *D4Mit6, Npy,* and *Spr* were selected and founded the congenic BB.SHR rat strain, which is normotensive (BB.SHR 118 ± 4 mm Hg; BB 119 ± 5 mm Hg BB).[4] To confirm the genetic background of BB/OK rats, BB.SHR rats (N6F1) were tested with an additional 86 microsatellite markers, which were not tested during creation of this congenic strain.

After weaning, up to an age of 7 weeks, 18 male BB/OK and 11 male BB.SHR rats were fed a commercial diet (RMH-B, Hope Farms BV, Woerden, The Netherlands). The chemical composition of this commercial diet has been described previously.[8] After this pre-experimental period, the rats were fed for 4 weeks the commercial diet supplemented with 5% (wt/wt) olive oil (Reddy, Van de Moortele NV, Oudenbosch, The Netherlands) and 2% (wt/wt) cholesterol (USP, Solvay-Pharmaceuticals BV, Weesp, The Netherlands). All rats analyzed were normoglycemic (Table 1). The rats were housed in groups of 3 animals in Macrolon type III cages (Ehret GmbH, Emmendingen, Germany) under strict hygienic conditions. All rats were free of major pathogens as described previously.[9] The rats had free access to food and tap water and were maintained at a 12 hours per day light regimen (5:00 AM to 5:00 PM). Body weight and food intake were recorded at the beginning (day 0; 7 weeks of age) and at the end (day 28; 11 weeks of age) of the experimental period.

Phenotypic Characterization

After a 16-hour fasting period at the beginning and at the end of the experimental period, blood samples were taken in random order from the rats by orbital puncture while they were under light anesthesia (Sevofluran, Abbott, Germany). Blood was collected in tubes without anticoagulant. To collect serum, the blood in the tubes was allowed to clot at room temperature. Serum was prepared by low-speed centrifugation (10 minutes, 3,000*g*, 4°C). Day 0-sera were stored at −70°C until use. Day 28 sera were cooled at 4°C and kept at that temperature until ultracentrifugation. Isolation of the lipoprotein fractions was performed within 5 days after blood sampling and was performed by density gradient ultracentrifugation as described,[10] except for staining. Lipoprotein density classes were based on the pattern observed in humans: very–low-density lipoprotein (VLDL) ($d < 1.006$), intermediate-density lipoprotein (IDL) ($1.006 < d < 1.019$), low-density lipoprotein (LDL)/high-density lipoprotein $(HDL)_1$ ($1.019 < d < 1.063$), HDL_2 ($1.063 < d < 1.125$), HDL_3 ($1.125 < d < 1.210$) and very–high-density lipoprotein (VHDL) ($d > 1.210$). Total serum triglycerides (day 0 and day 28), total serum (day 0 and day 28), and lipoprotein (day 28) cholesterol and phospholipid concentrations were determined in each individual rat using enzymatic kits (Boehringer Mannheim GmbH, Mannheimm, Germany) as described.[5,6] Lipoprotein cholesterol and phospholipid concentrations were corrected for recovery. Average recovery for serum cholesterol and phospholipid was 92% and 120%, respectively. Recovery is the sum of lipoprotein cholesterol or phospholipid as a percentage of total cholesterol or phospholipid in whole serum.

Serum insulin and leptin were determined using radioimmunoassay kits (Rat Insulin RIA Kit and Rat Leptin RIA Kit; Linco Research, St. Charles, MO).

Statistical Analysis

Results are presented as means ± SD. The Kolmogorov-Smirnov 1-sample test was used to check normality of the data. All results within the BB/OK or BB.SHR group were found to be normally distributed. The significance of differences between BB/OK and BB.SHR rats was calculated with the unpaired Student's *t* test. The unpaired Student's *t* tests were performed with pooled (for equal variances) or separate (for

unequal variances) variance estimates. The equality of variances was tested using an F test. The level of significance was preset at $P < .05$. Two-side probabilities were estimated throughout. All statistical analyses were performed according to Steel and Torrie[11] using a SPSS PC+ computer program (SPSS, Chicago, IL).[12]

RESULTS

Table 1 summarizes the results for body weight, food intake, blood glucose, serum insulin, and serum leptin levels. Body weight and food intake of BB/OK and BB.SHR rats were comparable at an age of 7 weeks, but after the high-fat, high-cholesterol diet (at 11 weeks of age), the BB.SHR rats were significantly heavier, and the food intake was significantly higher than in the BB/OK rats. No significant differences between BB/OK and BB.SHR were found for either basal or postdietary serum insulin concentrations. Basal serum leptin concentration was significantly higher in the BB/OK strain, but the postdietary serum leptin level did not show a significant difference between the strains.

Table 2 shows the measured serum lipid phenotypes of the 2 strains. Postdietary, but not basal, total serum triglyceride level was higher in the BB/OK strain when compared with that of the congenic BB.SHR strain. Significantly higher basal total serum cholesterol levels were observed in BB.SHR rats, but this difference disappeared after feeding with the test diet. On average, BB.SHR rats had 13% higher basal total serum phospholipid levels than BB/OK rats, but this effect of the chromosome 4 segment disappeared after 4 weeks on the high-fat, high-cholesterol diet.

The increase of about 250% and 216% of total serum cholesterol concentration in the BB/OK and BB.SHR strain after the high-fat, high-cholesterol diet was mainly due to an elevation of the VLDL cholesterol level; about 70% of the cholesterol is associated with this lipoprotein fraction. Except for the HDL_2 fraction, there were no statistically significant differences in lipoprotein cholesterol levels between BB/OK and BB.SHR rats. HDL_2 cholesterol concentration was elevated in the BB.SHR rats when compared with animals from the BB/OK strain. LDL/HDL_1 phospholipid concentration was higher in the BB/OK strain, whereas HDL_2 and HDL_3 were elevated in the BB.SHR strain when compared with the BB/OK-progenitor.

Table 1. Basal and Postdietary Body Weight, Food Intake, Blood Glucose, Serum Leptin, and Serum Insulin Levels of BB/OK and BB.SHR Rats

Measure	BB/OK	BB.SHR	Significance*
Body weight (g)			
Day 0 (7 weeks of age)	116 ± 8	116 ± 5	1.000
Day 28 (11 weeks of age)	242 ± 18	274 ± 17	<.001
Serum insulin concentration (ng/mL)			
Day 0 (7 weeks of age)	0.43 ± 0.12†	0.42 ± 0.15	.848
Day 28 (11 weeks of age)	0.84 ± 0.62	0.70 ± 0.66	.574
Serum leptin concentration (ng/mL)			
Day 0 (7 weeks of age)	1.40 ± 0.27	1.02 ± 0.22	<.001
Day 28 (11 weeks of age)	2.63 ± 0.66	2.61 ± 0.57	.937

NOTE. Results are expressed as means ± SD for 18 (BB/OK) or 11 (BB.SHR) animals per strain.

*P value of 2-tailed unpaired Student's t test.

†For one BB/OK animal, there was not sufficient serum for the determination of insulin at 7 weeks of age.

DISCUSSION

To confirm the existence of previously described QTLs for basal serum lipid phenotypes on a region of chromosome 4 flanked by the *D4Mit6* and *Spr* loci (spanning 12 cm), this segment of chromosome 4 was transferred from the SHR/Mol onto the genetic background of the BB/OK rat resulting in a congenic BB.SHR rat.[4] In the present study, we examined the effect of this region on (postdietary) serum lipid phenotypes after feeding BB/OK and BB.SHR rats a high-fat, high-cholesterol diet.

At the age of 11, but not of 7 weeks, body weight differed significantly between BB/OK and BB.SHR rats (Table 1). The difference in body weight at 11 weeks corroborates our previous work in which at 16 weeks BB.SHR rats were also heavier than BB/OK rats.[4] Therefore, it is not likely that this strain difference is due to the feeding of a high-fat high-cholesterol diet, but is rather age-dependent.

The serum leptin increasing action of high-fat feeding (Table 1) agrees with the investigations of Kowalska et al.[13] In mice, circulating leptin also increases by feeding a high-fat diet and with age.[14] Thus, the reported increase in serum leptin levels at 11 weeks of age is presumably due to both aging and the consumption of a high-fat, high-cholesterol diet. BB/OK rats have higher serum leptin levels than BB.SHR rats, but this strain effect lost the level of statistical significance at the end of the test period (Table 1). Because it is well-known that leptin overexpression reduces body weight via anorexic and thermogenic action,[15] it could be assumed that the lower body weight of BB/OK, when compared with BB.SHR rats at 11 weeks of age, might be due to the higher serum leptin concentration in BB/OK rats.

In comparison with other rat inbred strains (eg, SHR/Ola and BN-Lx/Cub; Bottger et al[5]) BB/OK and BB.SHR rats have much higher total serum cholesterol levels at an age of 7 weeks. These higher levels of total serum cholesterol (Table 2) are in line with the results reported by Patel et al.[16] These investigators showed that BB Wistar rats have 25% higher total serum cholesterol levels than age-matched or weight-matched control Wistar rats. Our present results and those of Patel et al[16] suggest that BB rats, in general, contain allelic variants of genetic factors that increase total serum cholesterol levels. Possibly, these genetic factors result in intestinal hypertrophy, which in turn, leads to increased small intestinal cholesterol synthesis and absorption. In rodent models of type 1 diabetes mellitus, cholesterol synthesis is indeed enhanced in the small intestine.[17]

Previous linkage studies using BB/OK and SHR/Mol have suggested that SHR rats possess a QTL on chromosome 4 region (flanked by *D4Mit6* and *Spr*) that increase basal total serum cholesterol levels.[2] In keeping with our earlier results,[4] the present findings confirm this again: congenic BB.SHR rats when compared with BB/OK rats have higher total serum cholesterol levels at 7 weeks of age (Table 2). For rats, the bulk

Table 2. Lipid Phenotypes in the BB.SHR Congenic Strain Compared With the BB/OK Progenitor

Phenotype	BB/OK	BB.SHR	Significance*
Total serum triglyceride concentration (mmol/L)			
Day 0 (7 weeks of age)	1.43 ± 0.26	1.49 ± 0.25	.596
Day 28 (11 weeks of age)	2.08 ± 1.24	0.82 ± 0.51	.003
Total serum cholesterol concentration (mmol/L)			
Day 0 (7 weeks of age)	2.94 ± 0.14	3.53 ± 0.26	<.001
Day 28 (11 weeks of age)	7.34 ± 3.31	7.62 ± 1.96	.770
Lipoprotein cholesterol concentration, day 28 (11 weeks of age; mmol/L)			
VLDL	5.25 ± 3.11 (69.6%)†	5.27 ± 1.65 (68.4%)	.985
IDL	0.50 ± 0.23 (7.2%)	0.62 ± 0.33 (7.9%)	.273
LDH/HDL_1	0.58 ± 0.38 (8.6%)	0.44 ± 0.12 (5.9%)	.166
HDL_2	0.71 ± 0.29 (10.4%)	0.97 ± 0.17 (13.6%)	.036
HDL_3	0.06 ± 0.02 (0.9%)	0.07 ± 0.03 (0.9%)	.397
VHDL	0.23 ± 0.08 (3.4%)	0.25 ± 0.07 (3.2%)	.669
Total serum phospholipid concentration (mmol/L)			
Day 0 (7 weeks of age)	2.32 ± 0.11	2.62 ± 0.12	<.001
Day 28 (11 weeks of age)	2.66 ± 0.63	2.51 ± 0.24	.379
Lipoprotein phospholipid concentration, day 28 (11 weeks of age; mmol/L)			
VLDL	1.29 ± 0.72 (46.6%)†	1.06 ± 0.15 (42.5%)	.185
IDL	0.12 ± 0.06 (4.8%)	0.13 ± 0.04 (5.0%)	.873
LDL/HDL_1	0.24 ± 0.19 (9.3%)	0.15 ± 0.03 (5.9%)	.049
HDL_2	0.38 ± 0.15 (15.0%)	0.52 ± 0.16 (20.8%)	.019
HDL_3	0.13 ± 0.02 (5.0%)	0.15 ± 0.03 (5.9%)	.037
VHDL	0.49 ± 0.11 (19.3%)	0.50 ± 0.09 (19.9%)	.801

NOTE. Results are expressed as means ± SD for 20 (BB/OK) or 11 (BB.SHR) animals per strain.

*P value of 2-tailed unpaired Student's t test.

†Values in parentheses are the mean relative cholesterol or phospholipid concentrations (percentage of total serum cholesterol or phospholipid level).

of basal total serum cholesterol is carried by the HDL_2-lipoprotein fraction.[5] Thus, it could be anticipated that BB/OK and BB.SHR rats differ mainly with respect to HDL_2 cholesterol level. The significant difference in HDL_2 cholesterol levels after feeding the high-fat, high-cholesterol diet between the 2 strains supports this reasoning (Table 2). Cholesterol and phospholipid concentrations in the serum are positively associated with each other.[18] Likewise, the relative distribution of phospholipid over the various lipoprotein fractions coincides with the relative distribution of cholesterol (Table 2).

When compared with the mean value of 12 other rat inbred strains,[8] the BB/OK and BB.SHR strains have about 2.6 times higher basal total serum triglyceride levels (Table 2). The hypertriglyceridemic status of BB rats has been known for a long time. Patel et al[16] described that BB Wistar rats have 2.3 times higher total serum triglyceride levels than age-matched and weight-matched control Wistar rats. The BB/OK strain responds to the diet with an increase in total serum triglyceride level, whereas the BB.SHR shows a decrease (Table 2). The reaction of the BB.SHR strain is the normal (hypo)triglyceridemic response to a high-fat, high-cholesterol diet. We have also documented this for 12 other normoglycemic rat inbred strains.[8] Furthermore, the hypertriglyceridemic response of the BB/OK strain to a high-fat, high-cholesterol diet is also in line with the literature, as it has been described that animal models for type 1 diabetes mellitus show hypertriglyceridemia after a high-fat and/or cholesterol-rich diet.[19]

The triglyceride results after the high-fat, high-cholesterol diet suggest that the SHR/Mol contains a QTL in the *D4Mit6-Spr* segment of chromosome 4 that lowers total serum triglyceride levels. We had already found evidence for this when we compared the SHR.BN-congenic strains with the SHR/Ola progenitor after feeding the rats a high-fat, high-cholesterol diet.[7] Transfer of the segment *Il6-Npy* of chromosome 4 from the BN strain into the genetic background of SHR/Ola caused a significant increase in total serum triglyceride level when the congenic strain was compared with the SHR/Ola.[7] The *Il6-Npy* segment partially overlaps the *D4Mit6-Npy-Spr* segment. Thus, taken together, the results indicate that the QTL should be located somewhere between *D4Mit6* and *Npy*. To prove this, it would be highly desirable to narrow down this region by fine mapping of congenics and by generation of next congenic sublines carrying different segments of the candidate region on chromosome.[4]

It can be concluded that in addition to the previously described differences between BB/OK and BB.SHR rats,[4] the results of this study clearly show the impact of genes, lying within the transferred segment, on serum lipid phenotypes after high-fat, high-cholesterol diet. Fine mapping and positional cloning of genes within this segment will lead to the identification of factors determining the differences in postdietary response between BB/OK and BB.SHR rats.

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