Original Articles

A 14-gene Region of Rat Chromosome 8 in SHR-Derived Polydactylous Congenic Substrain Affects Muscle-Specific Insulin Resistance, Dyslipidaemia and Visceral Adiposity

(metabolic syndrome / SHR / PD/Cub / congenic strain / ApoA5 / Plzf (Zbtb16))

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Abstract. The SHR and the PD/Cub are two established rodent models of human metabolic syndrome. Introgression of a ca 30 cM region of rat chromosome 8 from PD/Cub onto the genetic background of SHR was previously shown to influence several of the metabolic syndrome-related traits along with causing the PLS in the SHR-*Lx* congenic strain. In the process of identification of the causative alleles, we have produced several congenic sublines. The differential segment of SHR-*Lx* PD5 congenic substrain [SHR.PD(*D8Rat42-D8Arb23*)/Cub] spans approximately 1.4 Mb encompassing only 14 genes. When comparing the metabolic, morphometric and gene expression profiles of the SHR-*Lx* PD5 vs. SHR, the polydactyly and several distinct metabolic features observed in the original SHR-*Lx* congenic were still manifested, suggesting that the responsible genes were "trapped" within the relatively short differential segment of PD/Cub origin in SHR-*Lx* PD5. Particularly, the SHR-*Lx* PD5 displayed substantial reduction of insulin sensitivity confined to skeletal muscle. Among the candidate genes, the promyelocytic leukaemia zinc-finger *Plzf* (*Zbtb16*) transcription repressor is most likely responsible for the *Lx* mutation resulting in PLS and could also be involved in the alteration of metabolic pathways. The sequence analysis of the *Plzf* gene revealed a SNP leading to a threonine to serine substitution in SHR at aminoacid position 208 (T208S). In summary, we have isolated a 1.4 Mb genomic region syntenic to human chromosome 11q23, which, apart from causing polydactyly-luxate syndrome (PLS), affects total body weight, adiposity, lipid profile, insulin sensitivity of skeletal muscle and related gene expression as shown in the SHR-*Lx* PD5 congenic substrain.

The identification of the genes responsible for common multifactorial diseases such as insulin resistance, obesity, hypertension or dyslipidaemia is becoming one of the major themes in current genetic/genomic research. This is understandable given the great impact of the mentioned maladies on public health worldwide and the gloomy projections of their ever rising incidence and prevalence. Several major approaches have been pursued for the cause, including the hypothesis-driven candidate gene studies (association, knock-out, knock-down) and the hypothesis-free whole-genome scans (linkage, SNP, haplotypes). As a result, nearly all human chromosomes as well as those of prominent model species (mouse, rat) are covered with putative functional and positional candidates for the complex diseases (e.g. Šeda 2004; Perusse et al., 2005). However, the identification of causative alleles and validation of their (patho)physiological relevance for the disease in general human population proved to be a real challenge. As recently pointed out by Flint et al. (2005), the combination of genetically designed rodent strain sets and the arising high-throughput technologies and bioinformatics may lead to more effective QTL cloning in the 21st century.

Metabolic syndrome (MetS) is a prevalent human condition characterized by clustering of insulin resis-

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Abbreviations: AUC – area under the curve, BN – Brown Norway, LEW – Lewis, MetS – metabolic syndrome, OGTT – oral glucose tolerance test, PLS – polydactyly-luxate syndrome, PPARα – peroxisome proliferator-activated receptor alpha, QTL/QTN – quantitative trait locus/nucleotide, SD – Sprague-Dawley, SHR(SP) – spontaneously hypertensive (stroke-prone) rat, SNP – single nucleotide polymorphism, TG – triglyceride, UTR – untranslated region, WKY – Wistar Kyoto.

tance, dyslipidaemia, obesity, hypertension and procoagulant state. Mounting evidence supports the notion that the nature of the genetic component of MetS is rather oligogenic than polygenic with several major genes influencing the variability of multiple aspects of the syndrome. The support comes either from studies showing "clustering" of linkage signals for a number of independently assessed MetS-related phenotypes (Hamet et al., 2005; Šeda et al., 2005) or from studies finding linkage for the complete syndrome utilizing the principal component or factor analyses (Loos et al., 2003). In numerous human (e.g. Talmud et al., 2002; Bosse et al., 2004) and rodent (e.g. Klimeš et al., 2003; Wallis et al., 2004) linkage and association studies, different features of metabolic MetS were found to map to human chromosome 11q23, syntenic to rat chromosome 8q22. We have previously established a congenic strain SHR-*Lx* (Křen et al., 1997), in which the mentioned region was introgressed onto the spontaneously hypertensive rat (SHR) genetic background from the polydactylous rat (PD/Cub, Křen 1975), an inbred model of metabolic syndrome (Šedová et al., 2000; Šeda et al., 2005). Compared to the SHR progenitor, the SHR-*Lx* congenic displays significantly lower blood pressure and heart weight (Křen et al., 1997) but higher triglyceride and free fatty acid concentrations (Šeda, 2004; Šeda et al., 2005). In order to identify the alleles responsible for the observed metabolic profile, we have produced several congenic substrains of SHR-*Lx* using the marker-assisted approach. The newly derived congenic strain with the so far narrowest segment of PD/Cub origin was utilized in this study to verify the presence and scope of the phenotypic effects of the introgressed region. Because of the minute genetic difference between the progenitor SHR and the congenic SHR-*Lx* PD5, we employed a protocol including administration of dexamethasone (a potent trigger of insulin resistance) in order to "demask" the potential sensitizing effect of the introgressed chromosome 8 segment.

Material and Methods

Rat strains

The spontaneously hypertensive rat (SHR/OlaIpcv) was derived by recurrent selective breeding of Wistar rats by Japanese authors Okamoto and Aoki in Kyoto, Japan (1963). The SHR colony in Prague was originally obtained from the National Institutes of Health in USA > 25 years ago and since then it has been maintained by brother × sister mating at the Academy of Sciences of the Czech Republic, Prague.

The SHR.PD(*D8Rat42-D8Arb23*)/Cub (SHR-*Lx* PD5 hereafter) congenic strain was derived by introgressing the RNO8 differential segment of the PD/Cub origin onto SHR genetic background by narrowing down the segment present in the SHR-*Lx* congenic strain (Křen et al., 1997; Křenová et al., 2000). The original SHR-*Lx* derivation was described previously (Křen et al., 1997). In short, one of the recombinant inbred strains (BXH11) that inherited a large segment of chromosome 8 including the *Lx* mutation from the BN-*Lx* strain was used to introgress this region of chromosome 8 onto the SHR background by backcross breeding. After the equivalent of 12 generations of selective backcrossing to the SHR progenitor strain, the differential chromosome segment in the vicinity of *Lx* was fixed and maintained in the homozygous state by brother × sister mating and selective inbreeding of the polydactylous offspring. In order to determine the extent of the differential segment of SHR-*Lx* PD5 congenic strain precisely, we amplified microsatellite markers *D8Rat41, D8Rat42, D8Rat43, D1Rat405, D8Got72, D8Rat94, D8Arb23, D8Rat215, D8Mit2* and *D8Rat44*. Although the original SHR.BN-*Lx*/Cub(*D8Rat37-D8Mit2*)/Cub congenic strain was derived from BN-*Lx* congenic and SHR strains, the differential segment became much narrower by sequential backcross mating to SHR background (Křenová et al., 2000), and the congenic segment of RNO8 in the resulting SHR-*Lx* PD5 strain has no sequences of BN/Cub origin, as the congenic segment is shorter than that of the BN-*Lx* progenitor (Šeda et al., 2002).

Experimental protocol

All experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997), which is in compliance with the European Community Council recommendations for the use of laboratory animals 86/609/ECC, and were approved by the ethical committee of the First Faculty of Medicine. Male SHR (N = 13) and SHR-*Lx* (N = 10) rats were fed standard laboratory chow *ad libitum*. At the age of 5 months, dexamethasone (DEX, Dexamed, Medochemie, Ltd., Limasso, Cyprus) was administered in drinking water (0.026 mg/ml) for 3 days. Blood samples were drawn and the oral glucose tolerance test (OGTT) was performed before and after the DEX administration. Then the rats were sacrificed and the weights of heart, liver, kidneys, adrenals and epididymal and retroperitoneal fat pads were determined. The liver and heart tissues were snap frozen in liquid N_2 for gene expression analyses, the muscle and adipose tissues were used for *in vitro* assessment of insulin sensitivity.

Nucleic acid isolation, genotyping, sequencing, real-time PCR

DNA isolation, genotyping. The rat genomic DNA was isolated from the tail incision samples using a modified phenol extraction method. Polymorphic microsatellite loci were amplified by PCR using conditions optimized for each marker. Sequences of the selected markers were retrieved from public databases (Rat Genome Database, http://rgd.mcw.edu/, The Wellcome Trust Centre for

Human Genetics, http://www.well.ox.ac.uk/ or Whitehead Institute/MIT Center for Genome Research, http://www-genome.wi.mit.edu/). The PCR products were separated on polyacrylamide (7–10%) or agarose (2–4%) gels, stained by ethidium bromide and visualized using InstaDoc digital system (BioRad Laboratories, Hercules, CA).

DNA sequencing. A prediction of rat *Plzf* mRNA was derived from BLAST alignment of mouse and human *Plzf* mRNA to the rat genome (v3.1) using NCBI BLAST service. cDNA from adult hearts of rat inbred strains PD/Cub, BN-*Lx*/Cub, SHR-*Lx* PD5, SHR/OlaIpcv and BN/Cub was amplified with primers specific to predicted rat *Plzf* mRNA. Primers were designed using Primer3 (Rozen and Skaletsky, 2000). Genomic DNA obtained from tail biopsy of spontaneously hypertensive, stroke-prone rat (SHRSP), Wistar Kyoto (WKY), Sprague-Dawley (SD) and Lewis (LEW) rat inbred strains was amplified to obtain a segment of the first *Plzf* coding exon. PCR products were run on 1.5 % agarose in 1x TBE buffer and purified using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Sequencing was performed with BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), followed by ethanol precipitation of sequencing products, their resuspension in formamide, denaturation and electrophoresis in ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). Sequences were assembled into contigs using CAP (Huang, 1992) and different strains aligned using ClustalW (Chenna et al., 2003), in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) environment.

RNA isolation. Total RNA was isolated from the liver by the acid guanidinium thiocyanate-phenol-chloroform method using an RNA extraction solution (TRIZOL Reagent, Sigma, St. Louis, MO). RT-PCR was carried out using an RT-PCR kit (Sigma) according to manufacturer's instructions.

Real-time PCR. Real-time PCR assessment of the expression of selected genes (*Plzf, ApoA5, Pten, Hnf4, Fasn, Insig1*) in liver and heart tissues was performed using the SYBR green assay (QuantiTect™ SYBR® Green PCR kit, Qiagen) in Cepheid Smartcycler II according to manufacturer's instructions. Primers were designed not to produce identical products for genomic and cDNA. Primer sequences are shown in Table 3. Melting curve analysis was performed for each PCR reaction to verify specificity and identity of the PCR products.

Metabolic measurements

The oral glucose tolerance test (OGTT) was performed after overnight fasting. Blood for glycaemia determination was drawn from the tail at intervals of 0, 30, 60 and 120 minutes after the intragastric glucose administration to conscious rats (3 g/kg total body weight, 30% aqueous solution). The serum concentrations of triglycerides (TG), free fatty acids (FFA), insulin and blood glucose were determined as described previously (Šedová et al., 2000). In short, commercially available analytical kits were employed to determine blood glucose and serum triglyceride concentrations (Pliva-Lachema, Brno, Czech Republic). Serum FFAs were determined using an acyl-CoA oxidase-based colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany). Serum insulin concentration was determined using an ELISA kit for rat insulin assay (Mercodia, Uppsala, Sweden).

Insulin-stimulated glycogen synthesis. Basal and insulin-stimulated glucose incorporation into glycogen (conversion of [^{14}C]glucose to [^{14}C]glycogen) was determined in isolated soleus muscle as decribed previously (Vrána and Kazdová, 1970; Vrána et al., 1978; Šeda et al., 2003).

Insulin-stimulated lipogenesis. Basal and insulin-stimulated incorporation of ^{14}C-U glucose into total lipids of rat adipose tissue *in vitro* (lipogenesis) was determined. In short, after decapitation, distal parts of the epididymal adipose tissue (200 mg) were incubated in Krebs-Ringer bicarbonate buffer under conditions described above. Total adipose tissue lipids were extracted according to Folch et al. (1957) and the radioactivity was determined as described previously (Vrána and Kazdová, 1970).

Statistical analysis

When comparing more than two groups, one-way ANOVA was used with the post-hoc Tukey's honest significance difference test for comparison of the specific pairs of variables. For comparisons of only two groups, Student's t-test was used. Null hypothesis was rejected whenever $P < 0.05$.

Results

Fine mapping of the RNO8 differential segment in the SHR-Lx PD5 congenic strain

By densely genotyping the differential segment of chromosome 8 using the battery of microsatellite markers, the centromeric and telomeric markers of SHR origin in SHR-*Lx* PD5 congenic strain were found to be *D8Rat42* and *D8Arb23*, respectively (Fig. 1). The "maximal" differential segment therefore encompasses 1,446 kb (21,006–22,452 kb of rat chromosome 8 supercontig NW_047799). The "core" of the congenic segment (of PD/Cub origin) extends from *D1Rat405* to *D8Rat94* (513 kb). Although there is one marker, *D8Rat43*, between *D8Rat42* and *D1Rat405*, it is not polymorphic between SHR and PD/Cub. There are 14 annotated genes between *D8Rat42* and *D8Arb23* (Fig. 1).

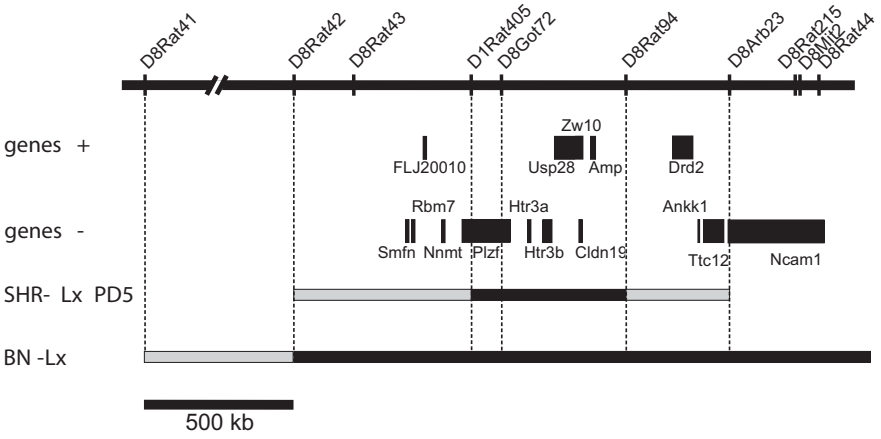


Fig. 1. Differential segment of chr. 8 in the SHR-Lx PD5 congenic strain
Upper track: physical map of the *Lx* locus on the rat chromosome 8. Microsatellite markers mapped in SHR.*Lx* PD5 and their relative distances based on the rat genome sequence are shown.
Medium track: genes expressed from + and complementary strands, represented as black boxes (exons and introns are not distinguished).
Lower tracks: differential segment of SHR.*Lx* PD5 congenic substrain, indicating the localization of *Lx*. Black line – segment of PD origin, grey line – uncertain genotype, the rest of the chromosome 8 is of SHR origin. For comparison, the extent of the differential segment in the BN-*Lx* congenic strain is indicated (Šeda et al., 2002).

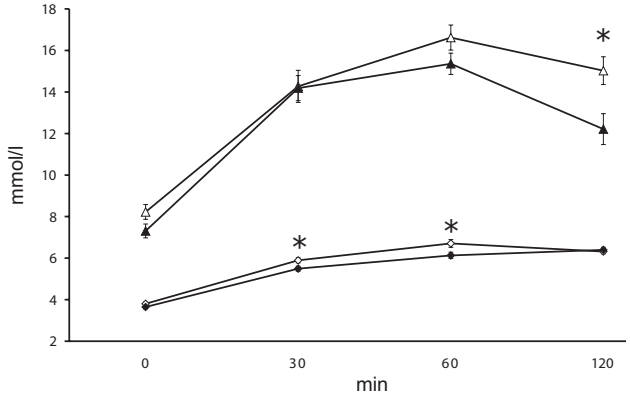


Fig. 2. Oral glucose tolerance test
Oral glucose tolerance test in SHR (open symbols) and SHR-*Lx* PD5 (full symbols) before and after dexamethasone administration (diamonds and triangles, respectively). Statistical significance levels are indicated as follows: *P < 0.05.

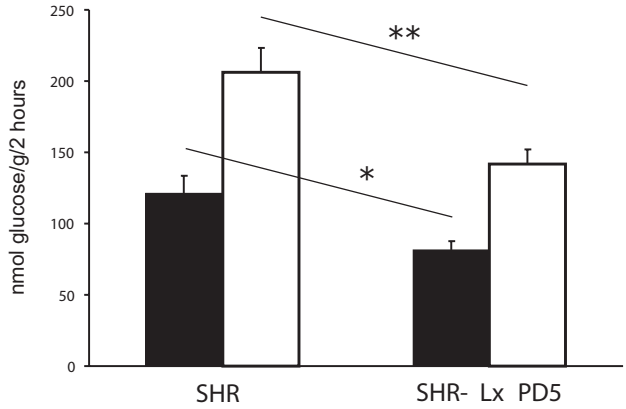


Fig. 3. Insulin-stimulated glucose utilization in muscle
Basal (full bars) **and insulin-stimulated** (open bars) **glucose incorporation into glycogen of *m. soleus*** in SHR and SHR-*Lx* PD5. Statistical significance levels are indicated as follows: *…P < 0.05, **…P < 0.01.

Morphometric and metabolic profile of SHR vs. SHR-Lx PD5

The SHR-*Lx* PD5 rats fully manifest the polydactyly-luxate syndrome in a form identical to all other SHR congenic strains carrying the *Lx* mutation, showing unaffected zeugopod with preaxial polydactyly of the hind feet. Compared to the SHR, the standard diet-fed SHR-*Lx* PD5 rats displayed lower total body weight (Table 1), lower postprandial triglyceride concentrations (Table 2) and a tendency towards an enhanced glucose tolerance (AUC of OGTT: 687.3 ± 12.6 vs. 725.1 ± 12.7 mmol/l/2 h in SHR-*Lx* PD5 and SHR, respectively; P = 0.05).

After the three-day dexamethasone challenge, both strains responded by similar relative weight loss, the SHR thus remaining significantly heavier. The SHR-*Lx* PD5 congenic had lower relative weights of adrenals and the epididymal (visceral) adipose tissue (Table 1). Although the glycaemia 2 h after the glucose instillation was significantly lower in SHR-*Lx* PD5 (Fig. 2), the overall glucose tolerance after the dexamethasone administration did not seem to differ between the strains (AUC of OGTT: 1593.4 ± 66.1 vs. 1755.1 ± 72.6 mmol/l/2 h in SHR-*Lx* PD5 and SHR, respectively; not significant). The insulin concentrations throughout the OGTT followed a similar pattern in both strains (data not shown). The postprandial TG showed a contrasting pattern to that observed under standard diet conditions: in response to dexamethasone, the triglyceride concentration has risen by 146% in SHR-*Lx* PD5 compared to 55% rise in SHR (Table 2), while a similar change and absolute values were observed in both strains for fasting TG. The insulin sensitivity of visceral adipose tissue was comparable in the two strains; however, both basal and insulin-stimulated glycogenesis was substantially impaired in SHR-*Lx* PD5 skeletal muscle in comparison to SHR (Fig. 3).

Sequence analysis of the Plzf (Zbtb16) gene

We obtained full coding sequences and partial 5' and 3' untranslated regions (UTRs) of the promyelocytic leukaemia zinc-finger (*Plzf* or *Zbtb16* - zinc finger and BTB domain-containing 16) transcript expressed in the heart of adult PD/Cub, BN/Cub and SHR/OlaIpcv (deposited under GenBank accession numbers: AY781102, AY781103 and AY781104, respectively). Sequence AY781102 is currently selected as the

Table 1. Morphometric profile of SHR and SHR-Lx

Trait	SHR	SHR-Lx PD5	P
Initial body weight, g	332.7 ± 5.0	294.4 ± 4.7	< 0.001
Final body weight, g	275.3 ± 4.0	250.9 ± 3.9	< 0.001
Liver wt., g/100g b. wt.	3.70 ± 0.08	3.92 ± 0.04	0.032
Heart wt., g/100g b. wt.	0.44 ± 0.01	0.44 ± 0.01	ns
Kidney wt., g/100g b. wt.	0.74 ± 0.01	0.76 ± 0.01	ns
Adrenals wt., mg/100g b. wt.	7.76 ± 0.29	6.52 ± 0.28	0.007
EFP wt., g/100g b. wt.	0.90 ± 0.03	0.79 ± 0.03	0.029
RFP wt., g/100g b. wt.	0.68 ± 0.09	0.57 ± 0.06	ns

Morphometric profile of SHR and SHR-*Lx* with P values for Student's t-test. Values are shown as mean ± S.E.M.; b.wt – body weight; EFP – epididymal fat pad; RFP – retroperitoneal fat pad.

Table 2. Metabolic profile and insulin sensitivity of peripheral tissues

Trait	Unit	SHR	SHR-Lx PD5	P
$Glucose_{STD}$ (f)	mmol/l	3.80 ± 0.09	3.65 ± 0.09	ns
$Triglycerides_{STD}$ (f)	mmol/l	0.58 ± 0.02	0.56 ± 0.01	ns
$Triglycerides_{STD}$ (nf)	mmol/l	0.73 ± 0.05	0.60 ± 0.02	0.037
$Glucose_{DEX}$ (f)	mmol/l	8.22 ± 0.35	7.31 ± 0.33	ns
$Insulin_{DEX}$ (f)	pmol/l	0.59 ± 0.09	0.57 ± 0.06	ns
$Insulin_{DEX}$ (nf)	pmol/l	1.51 ± 0.03	1.56 ± 0.03	ns
$Triglycerides_{DEX}$ (f)	mmol/l	0.98 ± 0.05	1.12 ± 0.09	ns
$Triglycerides_{DEX}$ (nf)	mmol/l	1.13 ± 0.07	1.48 ± 0.13	0.023
Free $fatty\ acids_{DEX}$ (f)	mmol/l	2.36 ± 0.05	2.39 ± 0.04	ns
Free $fatty\ acids_{DEX}$ (nf)	mmol/l	1.08 ± 0.06	1.09 ± 0.07	ns
Muscle triglycerides	μmol/g	1.79 ± 0.17	1.67 ± 0.14	ns
Lipogenesis (insulin –) *		18.2 ± 1.9	19.3 ± 1.9	ns
Lipogenesis (insulin +) *		32.1 ± 3.2	37.9 ± 2.8	ns
Glycogenesis (insulin –) #		121.0 ± 12.8	81.8 ± 5.8	0.019
Glycogenesis (insulin +) #		206.0 ± 17.5	141.9 ± 9.9	0.006

Summary of the metabolic profile and insulin-mediated glucose utilization in adipose and muscle tissues in SHR and SHR-*Lx* PD5 with P values for Student's t-test. Values are shown as mean ± S.E.M.; STD – standard diet; DEX – 3 days of dexamethasone administration; ns – not significant; * – nmol glucose/mg protein/2 h; # – nmol glucose/g/2 h. Methods abbreviated as Lipogenesis and Glycogenesis are described in the text.

Table 3. Primer sequences

Gene	Forward primer	Reverse primer
ApoA-5	5'-ctgtacaggcgaggaagagc-3'	5'-attccagtcaccaagcgttc-3'
Fasn	5'-agacccctgtggtgtttgag-3'	5'-ccctccagcatgtacacctt-3'
Hnf4	5'-ctgggtgtcagtgccctgtg-3'	5'-atggagggtaggctgctgtc-3'
Insig1	5'-cacgatcacgtctggagcta-3'	5'-cttgtgtggttctcccaggt-3'
Pten	5'-ggaaaggacggactggtgta-3'	5'-aggtttcctctggtcctggt-3'
PlzfC1	5'-gagatccggagcaacagttc-3'	5'-tcatcagatgcttggatggt-3'
PlzfC2	5'-aaagcagaggacctggatga-3'	5'-tccctcctgagacgctagac-3'
PlzfC3	5'-gcatatggagatgatgcaggt-3'	5'-accgttttccacagagttcg-3'
PlzfC4	5'-agtgtgtgtggggtcgaact-3'	5'-gcactcaaagggcttctcac-3'
PlzfC5	5'-gagcacactcaagagccaca-3'	5'-tccaaggccaaataacaacc-3'

The sequences of forward and reverse primers used in RT PCR of candidate genes and sequencing of the *Plzf* coding sequence (PlzfC1-C5). The primer pair PlzfC3 was used for assessment of the *Plzf* gene expression.

NCBI's reference sequence for *Plzf* (NM_001013181). We identified three SNPs, all in the first coding exon (Fig. 4). Two SNPs are silent, but one leads to a threonine to serine substitution in SHR at aminoacid position 208 (T208S). We resequenced full-length cds of BN-*Lx* and SHR-*Lx* PD5 and thus confirmed their identity with the PD/Cub sequence. In order to exploit the variability of these sequences in the rat, we resequenced a fragment of the first coding exon (exon 2) in four additional rat strains (SHRSP, SD, WKY and LEW, deposited under GenBank accession numbers: AY879593, AY879594, AY879595 and AY879596, respectively). We also compared the *Plzf* sequence of the rat with those of human, mouse, chicken and zebrafish. While humans and rat strains BN, LEW, SD, PD, BN-*Lx* and SHR-*Lx* PD5 have threonine at the position 208, mouse, chicken, zebrafish and rat strains SHR, SHRSP and WKY have serine at this position (Fig. 4).

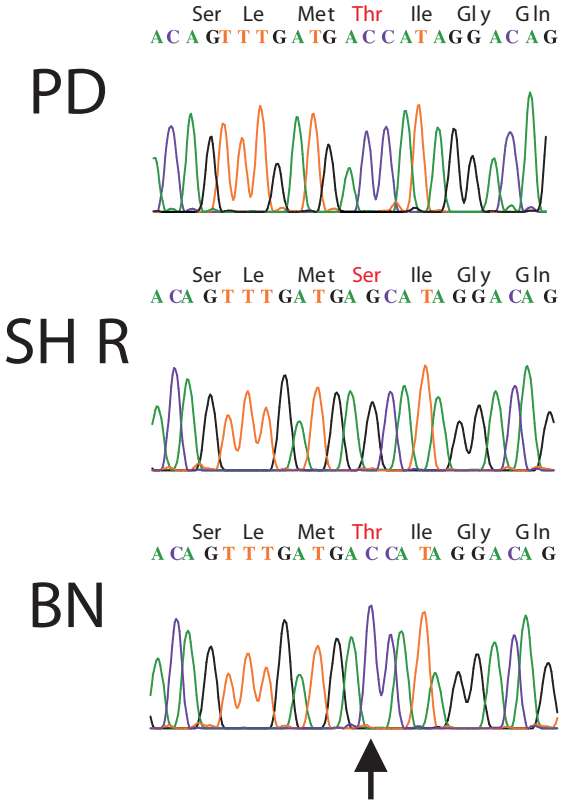
RT-PCR expression analysis

The real-time reverse-transcriptase PCR was performed in liver and heart for the set of candidate genes involved in major pathways affecting lipid and carbohydrate metabolism (Fig. 5). The apolipoprotein A5 and

A

position		BN	SHR	PD	BN.Lx	PD5	SHRSP	WKY	SD	LEW	change (BN ancestral)	
AY781103	AA										nt	AA
649	163	T	C	C	C	C	C	C	C	T	649T>C	G163G
783	208	C	G	C	C	C	G	G	C	C	783C>G	T208S
853	231	T	G	G	G	G	G	G	G	T	853T>G	G231G

B



C

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Rn  SHR  AAV54526   176 LPGPMVDQSPSVSTSFGLSAMSPTKAAVDSLMSIGQSLLQGALQPPAGPEEPTLAGGGR 234
Rn  BN   AAV54525   176 LPGPMVDQSPSVSTSFGLSAMSPTKAAVDSLMTIGQSLLQGALQPPAGPEEPTLAGGGR 234
Rn  PD   AAV54524   176 LPGPMVDQSPSVSTSFGLSAMSPTKAAVDSLMTIGQSLLQGALQPPAGPEEPTLAGGGR 234
Hs  NP_005997       176 LPGPMVDQSPSVSTSFGLSAMSPTKAAVDSLMTIGQSLLQGTLQPPAGPEEPTLAGGGR 234
Mm  AAB33814        176 LPGPMVDQSPSVSTSFGLSAMSPTKAAVDSLMSIGQSLLQGTLQPPAGPEEPTLAGGGR 234
Dr  NP_955929       177 LPGPMVDQSPSVSTSFGLSTMSPTKAAVDSLMSIGQSLLQSTMPPGVGAEQPLHGN--S 234
Gg  XP_417898       176 GPGAVVEQSPSVSTSFGLSAMSPTKAAVDSLMSIGQSLLQGALQHNVP-EEPHAAG--R 231
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Fig. 4. Sequencing of the *Plzf* gene and multiple species alignment of the Plzf protein
A. SNPs identified in the *Plzf* coding sequence and the haplotypes of various rat strains. The nucleotide position is annotated according to cDNA sequence AY781103 (strain BN/Cub), aminoacid positions according to protein AAV54525
B. Nucleotide sequence flanking the site of T208S substitution. The position of the substitution is indicated by an arrow, the polymorphic aminoacid is in red.
C. Multiple alignment of vertebrate Plzf proteins in comparison to rat variants at position 208. The hydroxyl moiety, but neither serine nor threonine alone, is conserved at this position of the Plzf protein among vertebrates.

hepatocyte nuclear factor 4 were the only genes showing significant differences, both being more expressed in liver of SHR-*Lx* PD5 compared to SHR. The expression of all other tested genes including the *Plzf* gene was found to be comparable in livers and hearts of dexamethasone-treated SHR and SHR-*Lx* PD5 rats (Fig. 5).

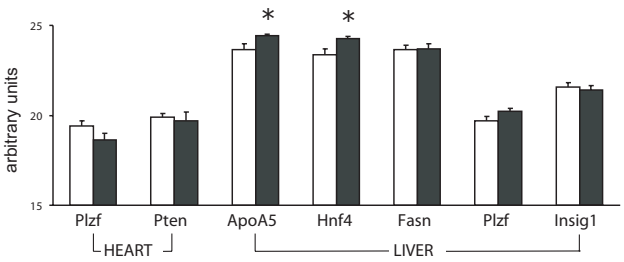

Fig. 5. Real-time PCR expression in liver and heart
Comparison of the level of expression of selected genes in SHR (open bars) and SHR-*Lx* PD5 (closed bars) heart and liver, assessed by real-time PCR. The tested genes were *Plzf*, phosphatase and tensin homologue (*Pten*), apolipoprotein A5 (*ApoA5*), hepatocyte nuclear factor 4 (*Hnf4*), fatty acid synthase (*Fasn*), insulin-induced gene 1 (*Insig1*). Statistical significance levels are indicated as follows: *…$P < 0.05$.

Discussion

One of the greatest challenges of the genetic analysis of complex traits is undoubtedly the path from obtaining evidence of trait linkage to a relatively wide genomic region (mostly in the form of a quantitative trait locus, QTL) to its verification in independent studies and its extent reduction to one amenable to systematic analysis. In the setting of genetically defined animal models, the derivation of congenic strains and testing the phenotypic impact of the transferred chromosomal segment still remains a fairly laborious, yet indispensable step on the way from QTL to QTN (quantitative trait nucleotide) as documented by several recent findings (Dutil et al., 2005; Garrett et al., 2005).

We established several congenic sublines of the original SHR-*Lx* congenic strain, new congenic substrain differing from its SHR progenitor by only a 14-gene segment, nevertheless showing decreased insulin sensitivity of skeletal muscle (considered as hallmark of metabolic syndrome) and a swift rise in postprandial TG after dexamethasone administration in combination with smaller visceral fat depots and total body weight. Notably, we observed these effects in comparison with SHR, an established model of both dyslipidaemia and insulin resistance (Pravenec et al., 2004, Hubner et al., 2005). Among the genes present in the SHR-*Lx* PD5 differential segment, the transcription factor *Plzf* stands out as a gene most likely responsible for the polydactyly-luxate syndrome, its role being strongly supported by a closely resembling phenotype induced by a knockout mutation of *Plzf* in the mouse (Barna et al., 2000). By its functional allocation at the intersections of metabolic and developmental pathways, the *Plzf* makes a good candidate even for the observed insulin resistant and dyslipidaemic phenotype. From the three identified SNPs in the *Plzf* coding sequence, only one results in aminoacid substitution – serine to threonine at position 208. This is a conservative substitution, as both aminoacids have the same functional moiety (hydroxyl group). Serine and threonine are thought to be interchangeable at most positions and if phosphorylated, they are substrates of the same protein kinases [Prosite motifs PS00004-6 (www.expasy.org/prosite)]. Indeed, we found both serine and threonine at the conserved position in different vertebrates and among rat strains (Fig. 4). Distribution of the alleles in rat strains seems to be independent of the metabolic profile (e.g. the same allele in insulin-resistant, dyslipidaemic PD/Cub versus insulin-sensitive, normolipidaemic BN/Cub for threonine, similarly SHR versus WKY for serine). The protein region flanking the substitution, downstream BTB/POZ and acidic domains, is moderately conserved, but is not recognized as a particular conserved domain. Further studies utilizing both versions of rat *Plzf* will be necessary to determine whether this minor difference is metabolically "active" or "silent".

Still, it is possible that the causal allele is either present in the regulatory sequences of *Plzf*, or within another gene in the segment. The latter possibility would be surprising at least as far as the polydactyly is concerned; on the other hand, one of the key features of the *Plzf* mutations in mice, the compromised fertility (Buaas et al., 2004; Costoya et al., 2004), is not present in any of the *Lx*-bearing animal strains. Some of the neighbouring genes were also implied to influence features of the metabolic syndrome, as is the case of the nicotinamide N-methyltransferase gene (*Nnmt*) proposed as a candidate gene for hyperhomocysteinaemia (Souto et al., 2005). The serotonin receptors *Htr3a* and *Htr3b* were shown to be involved in blood pressure control via centrally influencing the sympathoinhibitory mechanism (Ferreira et al., 2004), the sympathoadrenal axis being an important player (and, according to some authors, even crucial, e.g. Brunner et al., 2002) in the development of metabolic syndrome. Also, several polymorphisms of the dopamine D2 receptor (*Drd2*) have been proposed to play a role in obesity (Wang et al., 2001) and hypertension (Li et al., 2001).

Somewhat controversial is our finding of a higher expression of *ApoA5* in the SHR-*Lx* PD5 congenic together with higher postprandial TG. Apolipoprotein A5 is a recently added member of the apolipoprotein cluster on human chromosome 11q23 (Pennacchio et al., 2001; reviewed in Šeda and Šedová, 2003) and is considered to be one of the important determinants of plasma TG. The expression and circulation levels of ApoA5 were shown to be inversely correlated with plasma TG concentrations and are strongly enhanced

by agonists of peroxisome proliferator-activated receptor alpha (PPARα; Schultze et al., 2005). Interestingly, while the activation of PPARα by fibrates is considered the basis of their metabolically favourable actions (Haluzík and Haluzík, 2005), the PPARα-deficient mice were found to be resistant to dexamethasone-induced insulin resistance and hypertension (Bernal-Mizrachi et al., 2003). Therefore, there may be a pharmacogenetically specific PPARα-mediated increase in the *ApoA5* expression in SHR-*Lx* PD5 coinciding with a rise in triglycerides, resulting from modulation of *ApoA5*-independent pathways. Nevertheless, both effects seemingly converge to one or several of the genes present in the differential segment of the SHR-*Lx* PD5 congenic strain.

In summary, we have isolated a 1.4 Mb genomic segment syntenic to human chromosome 11q23, which, apart from causing the polydactyly-luxate syndrome, affects total body weight, adiposity, lipid profile, insulin sensitivity of skeletal muscle and related gene expression as shown in the SHR-*Lx* PD5 congenic substrain. Although the question whether the observed metabolic and morphological phenotypes are influenced by a dense clustering of distinct genes or by a pleiotropic effect of a single allelic variant still remains to be answered, the particular importance of this region for genetic determination of metabolic syndrome-related traits is strongly supported.

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