Original Article

Spontaneously Hypertensive Rat Chromosome 2 with Mutant Connexin 50 Triggers Divergent Effects on Metabolic Syndrome Components

(metabolic syndrome / connexin / animal models / congenic strain)

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Abstract. Metabolic syndrome is a frequent condition with multifactorial aetiology. Previous studies indicated the presence of genetic determinants of metabolic syndrome components on rat chromosome 2 (RNO2) and syntenic regions of the human genome. Our aim was to further explore these findings using novel rat models. We derived the BN-*Dca* and BN-*Lx.Dca* congenic strains by introgression of a limited RNO2 region from a spontaneously hypertensive rat strain carrying a mutation in the *Gja8* gene (SHR-*Dca*, dominant cataract) into the genomic background of Brown Norway strain and congenic strain BN-*Lx*, respectively. We compared morphometric, metabolic and cytokine profiles of adult male BN-*Lx*, BN-*Dca* and BN-*Lx.Dca* rats. We performed *in silico* comparison of the DNA sequences throughout RNO2 differential segments captured in the new congenic strains. Both BN-*Dca* and BN-*Lx.Dca* showed lower total triacylglycerols and cholesterol concentrations compared to BN-*Lx*. Fasting insulin in BN-*Dca* was higher than in BN-*Lx.Dca* and BN-*Lx*. Concentrations of several proinflammatory cytokines were elevated in the BN-*Dca* strain, including IL-1α, IL-1β, IFN-γ and MCP-1. *In silico* analyses revealed over 740 DNA variants between BN-*Lx* and SHR genomes within the differential segment of the congenic strains. We derived new congenic models that prove that a limited genomic region of SHR-*Dca* RNO2 significantly affects lipid levels and insulin sensitivity in a divergent fashion.

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Abbreviations: EPO – erythropoietin, GIP – gastric inhibitory polypeptide, GLP1 – glucagon-like polypeptide-1, G-CSF – granulocyte colony-stimulating factor, GM-CSF – granulocyte-macrophage colony-stimulating factor, HDL – high-density lipoprotein, IFN-γ – interferon γ, M-CSF – macrophage colony-stimulating factor, MCP 1 – monocyte chemotactic protein 1, MIP-1α – macrophage inflammatory protein 1α, MIP-3α – macrophage inflammatory protein 3α, PCR – polymerase chain reaction, PP – pancreatic polypeptide, PYY – protein tyrosine tyrosine, RNO2 – rat chromosome 2, SHR – spontaneously hypertensive rat, TNF-α – tumour necrosis factor α, VEGF – vascular endothelial growth factor.

Introduction

Obesity, hypertension, insulin resistance and dyslipidaemia are all multifactorial traits with high prevalence worldwide. Clustering of several of these conditions in an individual constitutes the metabolic syndrome. Clinical diagnosis of metabolic syndrome requires the presence of any three of the following criteria: waist circumference exceeding a geoethnical and sex-specific threshold, systolic blood pressure ≥ 135 mmHg and/or diastolic blood pressure ≥ 85 mmHg (or antihypertensive therapy), triglyceride concentration ≥ 1.7 mmol/l, high-density lipoprotein cholesterol < 1.0 or 1.3 mmol/l for men and women, respectively (or hypolipidaemic therapy), and fasting glucose > 5.6 mmol/l or glucose-lowering therapy (Alberti et al., 2009).

In-depth analysis of the genetic architecture of metabolic syndrome is a complex endeavour in the general human population given the many factors that are not easily accounted for, including genome-environmental (Seda et al., 2008a) and higher complexity-level interac-

tions (Nikpay et al., 2012; Civelek and Lusis, 2014). One of the routes shown to yield relevant results with translational impact is the derivation and subsequent analysis of genetically designed rodent models of human complex conditions such as metabolic syndrome. The rat has been in the forefront of the physiological and pharmacological studies (Aitman et al., 2016) and is likely to keep its position even in the era of integrative genomics and system biology approaches (Moreno-Moral and Petretto, 2016). The reference rat system for genetics of the metabolic syndrome is the HXB BXH recombinant inbred rat panel, derived from the spontaneously hypertensive rat (SHR) strain and its normotensive counterpart, the Brown Norway BN-*Lx* congenic strain (Pravenec et al., 1989). One of the regions repeatedly showing linkage to metabolic syndrome components maps to the telomeric part of rat chromosome 2 (Alemayehu et al., 2002; Wallace et al., 2004; Seda et al., 2006; Graham et al., 2007; Chauvet et al., 2009).

We have previously derived the BN-*Lx*.SHR2 double-congenic rat strain that exhibits a distinct combination of dyslipidaemia and mild glucose intolerance in a non-obese setting, showing involvement of chromosome 2 alleles of spontaneously hypertensive rat in other than haemodynamic traits (Seda et al., 2006). Also, we have shown that mutation L7Q in connexin 50 (coded for by the *Gja8* gene) within the same chromosome 2 region in the SHR-*Dca* (*Dca* – dominant cataract) strain (Liska et al., 2008) decreases blood pressure, high-density lipoprotein (HDL) cholesterol and basal insulin sensitivity in skeletal muscle of the SHR (Šeda et al., 2017) and substantially affects the oxidative state parameters (Seda et al., 2016). The aim of the current study was to further explore the role of the region of rat chromosome 2 including the mutated *Gja8* gene in metabolic syndrome features in a comparative genomic perspective with the syntenic regions of the human genome.

Material and Methods

Ethical statement

The study was conducted in accordance with the Animal Protection Law of the Czech Republic and was approved by the ethical committee of the First Faculty of Medicine, Charles University. Animals were held under temperature and humidity controlled conditions in a 12-h light/dark cycle. At all times, the animals had free access to food (standard chow) and water.

Derivation of the BN-Dca and BN-Lx.Dca congenic strains

The BN/Cub [Rat Genome Database (Shimoyama et al., 2015) (RGD) RGD ID No. 737899], BN-*Lx*/Cub (BN-*Lx* hereafter; RGD ID No. 61117) and SHR-Gja8^{m1Cub} (SHR-*Dca* hereafter; RGD ID No. 2293729) strains are maintained at the Institute of Medical Biology and Genetics, Charles University in Prague. In order to derive the BN-*Dca* congenic strain and the BN-*Lx.Dca* double-congenic strain, we employed the marker-assisted backcross breeding approach as described previously (Seda et al., 2002; Šedová et al., 2012; Sedova et al., 2016). In short, the SHR-*Dca* rats were crossed with the other progenitor strain, i.e., BN/Cub or BN-*Lx*, for derivation of BN-*Dca* and BN-*Lx.Dca*, respectively. Subsequently, the F1 hybrids were repeatedly backcrossed to BN/Cub or BN-*Lx*. Then, we fixed the differential segment in each strain by intercrossing heterozygotes and selected the progeny inheriting the SHR-*Dca*-derived chromosome 2 segment in homozygous state. We validated the congenic status of the new BN-*Dca* and BN-*Lx.Dca* strains by a whole-genomic marker scan. Then, we precisely defined the extent of SHR-*Dca*-derived regions in both BN-Dca and BN-*Lx.Dca* by genotyping a set of 38 polymorphic chromosome 2 microsatellite markers.

Experimental protocol

At the age of four months, males of the new congenic strains (BN-*Dca*, N = 6; BN-*Lx.Dca*, N = 6) and the parental strain BN-*Lx* (N = 6) were subjected to oral glucose tolerance test after overnight fasting, and blood samples for other metabolic measurements were drawn. Then, the animals were sacrificed and their total weight and weight of the heart, liver, kidneys, adrenals, epididymal and retroperitoneal fat pads were determined.

DNA extraction, genotyping

The rat DNA was isolated by a modified phenol extraction method from tail incision samples. Nucleotide sequences of primers were obtained from public databases (RGD, http://rgd.mcw.edu/, The Welcome 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/). Polymerase chain reaction (PCR) was used for genotyping markers polymorphic between the progenitor strains. We tested DNA of both congenic strains (BN-*Dca*, N = 8; BN-*Lx.Dca*, N = 8) and the progenitor strains BN/Cub, BN-*Lx* and SHR-*Dca*. The PCR products were separated in polyacrylamide (7–10 %) gels, detected in UV light after ethidium-bromide staining using Syngene G:Box (Synoptics, Ltd., Cambridge, UK).

Metabolic measurements

Adult, standard chow-fed males (4 months of age) of all strains (N = 6/strain) were used for the metabolic measurements. The oral glucose tolerance test was performed after an overnight fast, and blood samples were taken for glycaemic determination (Ascensia Elite Blood Glucose Meter; Bayer HealthCare, Mishawaka, IN; validated by the Institute of Clinical Biochemistry and Laboratory Diagnostics of the First Faculty of Medicine) from the tail vein at intervals of 0, 30, 60, 120, and 180 min after intragastric glucose administration to conscious rats (3 g/kg body weight, 30% aqueous solution). Serum triacylglycerol and cholesterol concen-

trations were measured by standard enzymatic methods (Erba-Lachema, Brno, Czech Republic). Serum free fatty acid levels were determined using an acyl-CoA oxidase-based colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany). The Milliplex Rat Metabolic Hormone Magnetic Bead panel (Merck Millipore, Darmstadt, Germany) was used for simultaneous quantification of C-peptide, gastric inhibitory polypeptide (GIP), glucagon-like polypeptide-1 (GLP1), pancreatic polypeptide (PP), protein tyrosine tyrosine (PYY), glucagon, insulin and leptin; the Bio-Plex Pro Rat Cytokine 24-Plex Immunoassay (Bio-Rad, Hercules, CA) was used to assess the concentrations of erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-X-C motif) ligand 1 (GRO/KC), interferon γ (IFN-γ), interleukins IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, IL-18, macrophage colony-stimulating factor (M-CSF), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), macrophage inflammatory protein 3α (MIP-3α), regulated on activation, normal T cell expressed and secreted (RANTES), tumour necrosis factor α (TNF-α) and vascular endothelial growth factor (VEGF), using the BioPlex system (Bio-Rad).

Statistical analysis

All statistical analyses were performed using STATISTICA 12 CZ. The metabolic and morphometric data were compared by one-way analyses of variance (ANOVA) with STRAIN as the main factor followed by post-hoc Tukey's honest significance difference test for detailed pair-wise comparisons. The null hypothesis was rejected whenever $P < 0.05$.

In silico analyses

To compare the publicly available DNA sequences of SHR/OlaIpcv and BN-*Lx*, we utilized the Variant Visualizer resource provided by the Rat Genome Database (http://rgd.mcw.edu/rgdweb/front/select.html) with settings of high conservation (0.75–1.00) according to PHAST (http://compgen.cshl.edu/phast/) (Hubisz et al., 2011), minimum read depth set to 8 and exclusion of variants found in less than 15 % of reads. The results were then verified in the relevant NCBI-based databases. In order to identify the regions of the human genome syntenic to the differential segment ascertained in BN-*Dca* and BN-*Lx.Dca* congenic strains, we utilized the Virtual Comparative Map software tool (http://www.animalgenome.org/VCmap/). Then we overlapped the ascertained human genome regions with genomic positions of the significant loci reported in human genome-wide association studies (extracted from the Catalog of Published Genome-Wide Association Studies, available at: http://www.ebi.ac.uk/gwas/ (Welter et al., 2014), accessed on September 26th, 2016).

Results

Genomic characterization of the BN-Dca and BN-Lx.Dca congenic strains

The chromosome 8 differential segment of BN-*Lx* (PD/Cub) origin (Seda et al., 2002) in the BN-*Lx.Dca* double-congenic strain corresponded fully to that present in BN-*Lx*, delineated by markers D8Rat39 and D8Mgh6 (Fig. 1). Our genotyping scan using markers polymorphic between SHR and BN-*Lx* on chromosome 2 revealed the extent of differential segments of SHR-*Dca* origin in the BN-*Dca* and BN-*Lx.Dca* congenic

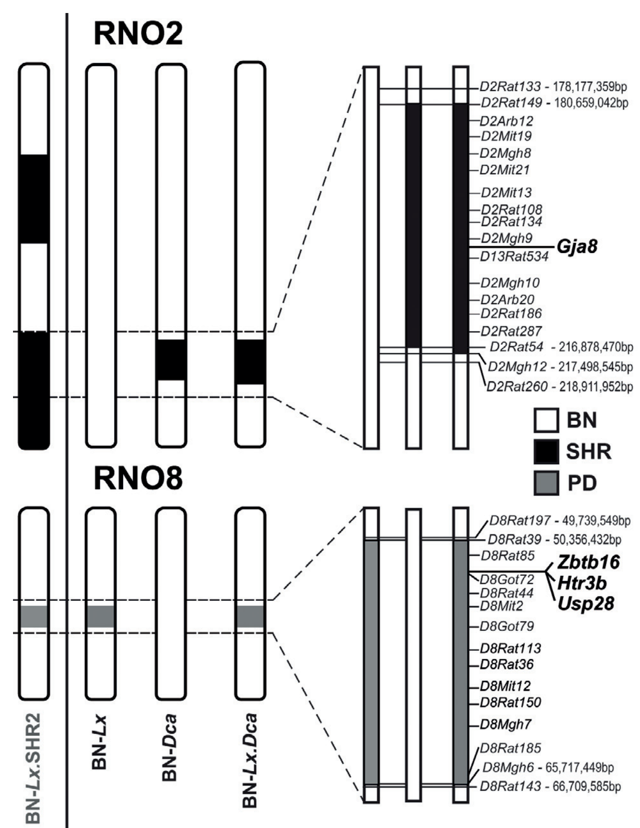

Fig. 1. The rat chromosome 2 (RNO2) and chromosome 8 (RNO8) differential segments in BN-*Lx*, BN-*Dca* and BN-*Lx.Dca* congenic strains. A subset of markers genotyped in this study to determine the differential segments is shown to the right of respective chromosomes. Open bars depict the chromosomal regions of BN origin, the SHR-*Dca*-derived segments of RNO2 are shown as black bars, the PD/Cub-derived segments of RNO8 are shown as grey bars. Genomic positions of markers spanning the respective differential segments are shown according to the *Rattus norvegicus* reference genome assembly Rnor_6.0. The genes with validated missense mutations in the congenic strains are shown to the right of the differential segments. To the left of the strains used in this study, the extent of differential segments in the BN-*Lx*.SHR2 congenic strain (Seda et al., 2006) is shown for reference.

Table 1. Morphometric comparison of BN-Lx, BN-Lx.Dca and BN-Dca male rats

Trait	BN-*Lx*	BN-*Lx.Dca*	BN-*Dca*	P
Body weight (b.wt.), g	242 ± 6	232 ± 4	246 ± 8	0.27
Liver, g/100 g b.wt.	2.27 ± 0.02	2.36 ± 0.03	2.39 ± 0.04	0.07
Heart, g/100 g b.wt.	0.29 ± 0.01	0.30 ± 0.01	0.30 ± 0.01	0.19
Kidney, g/100 g b.wt.	0.57 ± 0.01†	0.58 ± 0.01*	0.60 ± 0.01* †	**0.004**
Adrenals, mg/100 g b.wt	20.1 ± 0.9*	17.9 ± 0.3*	19.1 ± 0.4	**0.032**
EFP, g/100 g b.wt.	0.60 ± 0.05	0.64 ± 0.02	0.70 ± 0.03	0.09
RFP, g/100 g b.wt.	0.15 ± 0.04	0.21 ± 0.02	0.21 ± 0.02	0.11

Morphometric profile of BN-*Lx*, BN-*Lx.Dca* and BN-*Dca* male rats (N = 6/strain). Data are shown as mean ± S.E.M. The significance levels of one-way ANOVA for STRAIN as a major factor are shown in the last column. The significance levels for pair-wise, inter-strain comparisons between BN-*Lx*, BN-*Dca* and BN-*Lx.Dca* strains are shown for post-hoc Tukey's HSD test. Within a line, two values sharing the same superscript index differ significantly at the respective levels of P value as follows: * $P < 0.05$ and † $P < 0.01$, respectively; b.wt. – body weight; EFP – epididymal fat pad; RFP – retroperitoneal fat pad.

strains. The segments span about 38 Mb and 39 Mb in BN-*Dca* and BN-*Lx.Dca*, respectively. While the centromeric delimiting point is identical in both strains, the telomeric end of the differential segment stretches for about one more megabase in BN-*Lx.Dca* (Fig. 1). Several total genome scans conducted throughout the strains' derivation eventually excluded presence of other non-SHR-*Dca* alleles than those fixed on chromosome 2, confirming the congenic status of the new strains. The SHR-*Dca*-derived chromosome 2 segment hence represents the only genomic difference between BN-*Lx.Dca* and BN-*Lx* strains, and the chromosome 8 differential segment together with 1 Mb of chromosome 2 represent the only genomic differences between the BN-*Lx.Dca* and BN-*Dca* strains.

Morphometric and metabolic profile

The body weights as well as the weights of the heart, liver and adipose tissue depots were comparable among the three strains. BN-*Dca* had the highest relative kidney weight of all strains (Table 1). Double-congenic strain BN-*Lx.Dca* had lower adrenal gland weight compared to BN-*Lx*. Both BN-*Dca* and BN-*Lx.Dca* showed substantially lower total triacylglycerols and cholesterol concentrations compared to BN-*Lx*. Fasting free fatty acids and pancreatic polypeptide were higher in BN-*Dca* than in BN-*Lx* (Table 2). The overall glucose tolerance estimated by the area under the glycaemic curve did not differ among the strains, although the individual curves followed rather distinct courses. In the first 30 min of OGTT, glucose concentrations rose more substantially in BN-*Dca* and BN-*Lx.Dca* compared to BN-*Lx* (Fig. 2). Then, BN-*Dca*'s glycaemia decreased most rapidly, reaching the significantly lowest values of the three tested strains at 120th min. At the same time, the fasting insulin in BN-*Dca* was about two- to three-fold higher than in BN-*Lx.Dca* and BN-*Lx*, respectively. BN-*Lx* showed the lowest concentrations of leptin compared to both BN-*Dca* and BN-*Lx.Dca* strains (Table 2).

Table 2. Metabolic comparison of BN-Lx, BN-Lx.Dca and BN-Dca male rats

Trait	BN-*Lx*	BN-*Lx.Dca*	BN-*Dca*	P
Triacylglycerols, mmol/l	0.50 ± 0.03†^{c}	0.29 ± 0.01^{c}	0.33 ± 0.03†	**0.004**
Cholesterol, mmol/l	1.46 ± 0.06‡^{c}	0.72 ± 0.06^{c}	0.84 ± 0.09‡	**< 0.001**
Free fatty acids, mmol/l	0.67 ± 0.07*	0.86 ± 0.08	1.01 ± 0.10*	**0.033**
Insulin, pg/ml	59 ± 12‡	79 ± 17^{c}	148 ± 18 ‡^{c}	**0.012**
C-peptide, pg/ml	110 ± 15	125 ± 9	157 ± 11	0.08
GIP, pg/ml	133 ± 24	113 ± 9	143 ± 8	0.53
GLP1, pg/ml	158 ± 57	187 ± 30	209 ± 75	0.83
Glucagon, pg/ml	33 ± 3	33 ± 4	32 ± 6	0.96
PP, pg/ml	151 ± 44*	766 ± 370	1652 ± 512*	**0.037**
PYY, pg/ml	124 ± 35	738 ± 364	649 ± 321	0.33
Leptin, pg/ml	813 ± 28‡^{c}	4213 ± 212^{c}	3868 ± 453‡	**0.001**

Metabolic profile of BN-*Lx*, BN-*Lx.Dca* and BN-*Dca* male rats. Data are shown as mean ± S.E.M. The significance levels of one-way ANOVA for STRAIN as a major factor are shown in the last column. The significance levels for pair-wise, inter-strain comparisons between BN-*Lx*, BN-*Dca* and BN-*Lx.Dca* strains are shown for post-hoc Tukey's HSD test. Within a line, two values sharing the same superscript index differ significantly at the respective levels of P value as follows: * $P < 0.05$; † $P < 0.01$ and ‡, ^{c} $P < 0.001$, respectively.

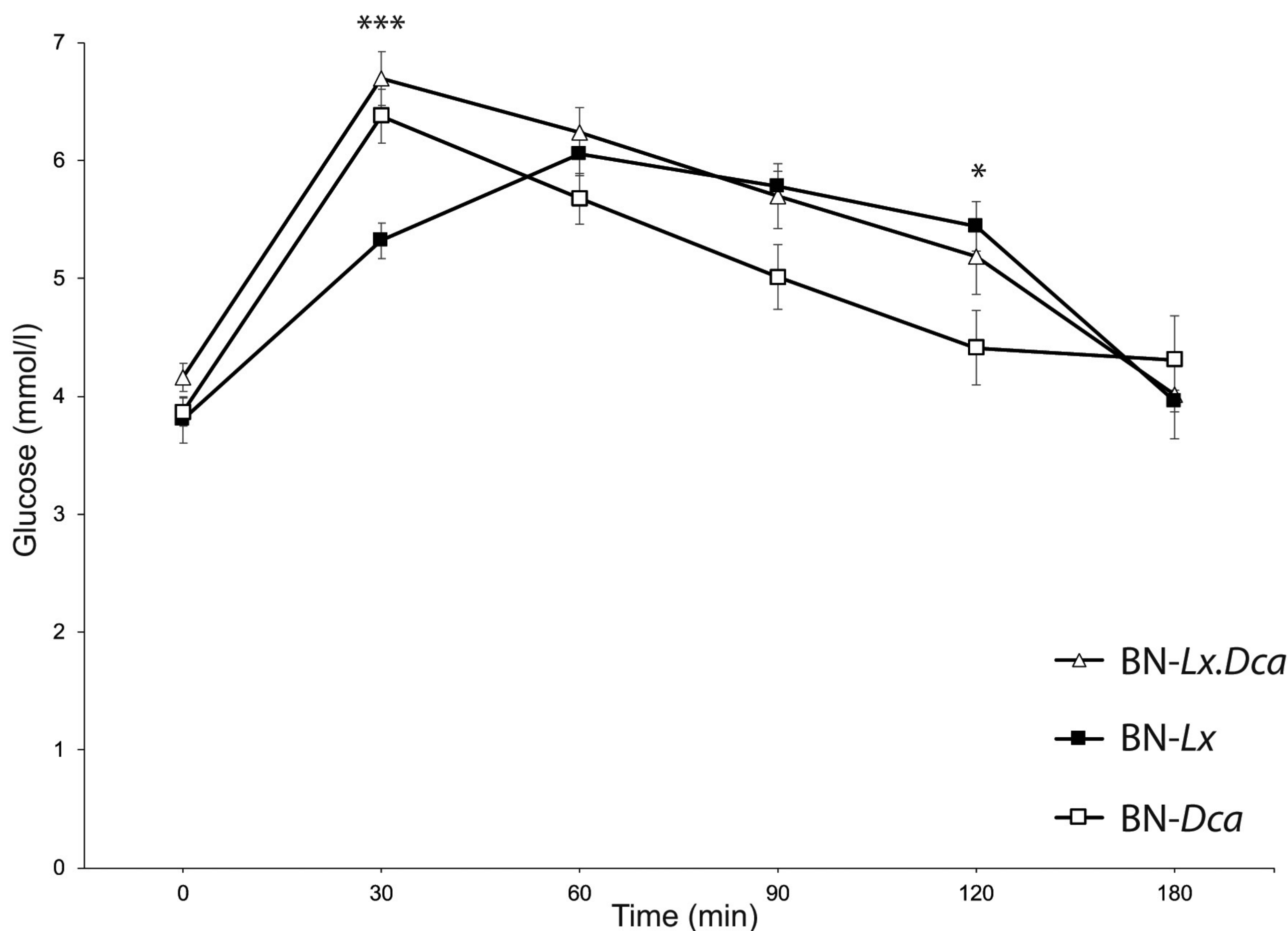

Fig. 2. The course of glycaemic curves in BN-*Lx* (black squares), BN-*Dca* (white squares) and BN-*Lx.Dca* (white triangles) male rats during the oral glucose tolerance test. Data are expressed as mean ± S.E.M. Significance levels for OGTT are given for factor STRAIN of one-way ANOVA as follows: * $P < 0.05$; *** $P < 0.001$.

Cytokine profile

Concentrations of several proinflammatory cytokines were elevated in the BN-*Dca* strain, which showed the overall highest concentration of IL-1α (BN-*Dca* vs. BN-*Lx* post-hoc Tukey's HSD P = 0.0097; BN-*Dca* vs. BN-*Lx.Dca* P = 0.0071) and increased levels of IL-1β compared to BN-*Lx* (post-hoc Tukey's HSD P = 0.0040) and of IFN-γ and MCP-1 compared to BN-*Lx.Dca* (post-hoc Tukey's HSD P = 0.0108 for IFN-γ and P = 0.0101 for MCP-1, respectively). No differences among the strains were found in concentrations of the other 20 tested cytokines (Fig. 3).

Prioritization of RNO2 candidate genes

The differential segments of BN-*Dca* and BN-*Lx.Dca* congenic strains contain 748 and 752 annotated genes (NCBI *Rattus norvegicus* Annotation Release 105, Rnor_6.0 assembly), respectively. We compared the genomic DNA sequences throughout the differential segments of the RNO2 congenic strains between the two parental strains *in silico* to identify highly conserved variations between SHR and BN-*Lx* genome sequences. In addition to the L7Q mutation in the *Gja8* gene distinguishing SHR-*Dca* from the SHR strain, we identified 34 protein-coding genes within the segment of both new strains predicted to carry non-synonymous mutations, seven of which anticipated to be probably damaging by the Polyphen prediction tool (Table 3). It is obvious that DNA variations other than the non-synonymous mutations may be responsible for the observed phenotypic effects. We therefore overlapped the information coming from the human genome-wide studies with DNA sequence variation between SHR and BN-*Lx* and in this manner identified polymorphisms in genes within the BN-*Dca* and BN-*Lx.Dca* RNO2 differential segments previously linked to the features of metabolic syndrome in man relevant to the findings of this study. Altogether, there were 27 genes on human chromosome 1 and one gene on human chromosome 4 with highly significant associations in human studies and at the same time showing one or more sequence variants in SHR (Table 4). The genes summarized in Tables 3 and 4 are the primary candidates for the observed effects of the chromosome 2 segment of SHR-*Dca* origin present in the two congenic strains.

Discussion

The two novel congenic models show that a limited genomic region of spontaneously hypertensive rat RNO2

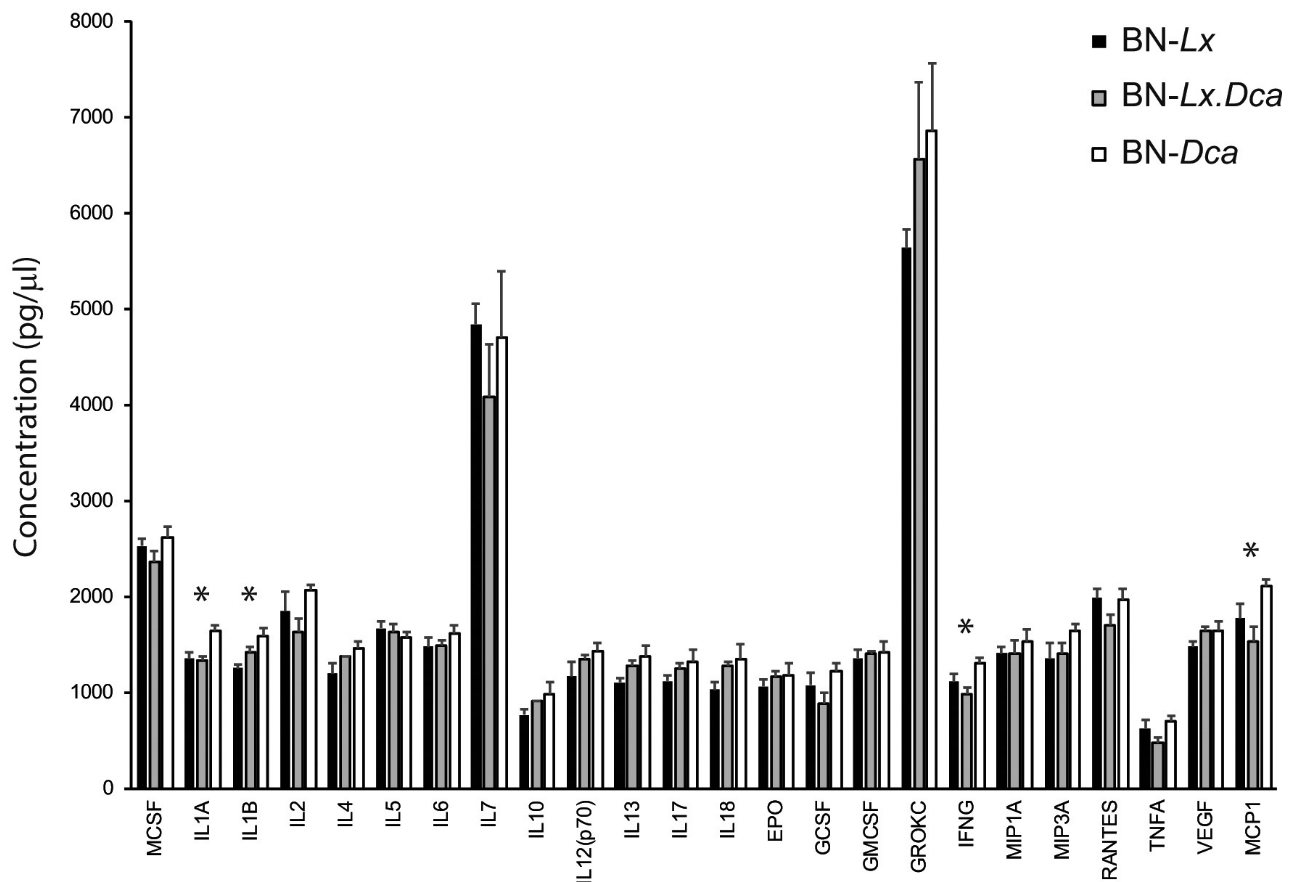

Fig. 3. Cytokine concentrations in the serum of BN-*Lx* (black bars) vs. BN-*Lx.Dca* (grey bars) and BN-*Dca* (open bars) adult male rats. Data are expressed as mean ± SEM. Within the graph, the significance levels of ANOVA for STRAIN as major factor are indicated as follows: * P < 0.05. GRO/KC – chemokine (C-X-C motif) ligand 1

significantly and divergently affects several features of metabolic syndrome. Compared to the previously published BN-*Lx*.SHR2 congenic strain (Seda et al., 2006) showing the impact of SHR chromosome 2 on lipid levels and glucose tolerance, the size of the differential segment in the current study is substantially reduced and a point mutation in the *Cx50* gene is added. In comparative genomic perspective, the captured region is syntenic to parts of human chromosomes 1 and 4. The differential segment overlaps numerous quantitative trait loci from experimental model studies involving SHR and BN rat strains focused on genetic mapping of metabolic syndrome-related parameters, including hypertension (Pravenec et al., 2001; Alemayehu et al., 2002), glucose intolerance and insulin secretion (Wallace et al., 2004) or lipid profile (Seda et al., 2006). Also, a number of significant genome-wide associations to metabolic syndrome features distinct in BN-*Dca* vs. BN-*Lx.Dca*

Table 3. Probably damaging DNA variants between SHR and BN-Lx

Gene Symbol	SNP ID	BN-*Lx* allele	SHR allele	BN-*Lx* AA	SHR AA
Arhgef11	rs198712982	G	A	Ala	Thr
Ttc24	rs198395321	C	A	Ala	Ser
Ttc24	rs198535348	G	A	Arg	Cys
Sprr3	rs199089885	A	G	Lys	Glu
Lingo4	rs197323120	G	A	Asp	Asn
Itga10	rs199015249	G	A	Gly	Asp
Mybphl	rs198291456	G	T	Trp	Leu

DNA variants between SHR and BN-*Lx* within the differential segment of the BN-*Dca* and BN-*Lx.Dca* congenic strains leading to non-synonymous mutations predicted to be probably damaging by the Polyphen prediction tool. SNP ID: identification of the single-nucleotide polymorphism according to NCBI's dbSNP database. AA: amino acid is indicated for BN-*Lx* and SHR strains; Arhgef11: Rho guanine nucleotide exchange factor 11; Ttc24: tetratricopeptide repeat domain 24; Sprr3: small proline rich protein 3; Lingo4: leucine rich repeat and Ig domain containing 4; Itga10: integrin subunit α 10; Mybphl: myosin binding protein H like.

Table 4. Genome-wide association studies in human subjects: reported genome-wide significant associations

Gene	HSA	Cholesterol	Insulin sensitivity	MCP1	SHR/BN-Lx variants
CELSR2	1	TC, LDL-C, HDL-C			2 (2/0/0)
MYBPHL	1	TC, LDL-C, HDL-C			2 (1/1/0)
SORT1	1	TC, LDL-C, HDL-C			4 (1/2/1)
PSMA5	1	TC			1 (0/1/0)
ATXN7L2	1	TC			1 (0/1/0)
GPR61	1	TC			2 (1/1/0)
GNAI3	1	TC			1 (0/1/0)
CSF1	1		QUICKI		1 (0/1/0)
AHCYL1	1		QUICKI		10 (0/9/1)
NOTCH2	1		T2D		11(4/7/0)
BCL9	1		T2D		4 (2/2/0)
SETDB1	1	LDL-C			1 (1/0/0)
CERS2	1	LDL-C			4 (3/1/0)
ANXA9	1	LDL-C			2 (2/0/0)
BNIPL	1	LDL-C			1 (1/0/0)
CDC42SE1	1	LDL-C			3 (0/3/0)
MLLT11	1	LDL-C			1 (1/0/0)
GABPB2	1	LDL-C			1 (1/0/0)
PMVK	1	HDL-C			4 (2/2/0)
DCST2	1			MCP 1	2 (2/0/0)
ADAM15	1			MCP 1	4 (2/2/0)
TRIM46	1			MCP 1	2 (1/1/0)
THBS3	1			MCP 1	3 (0/1/2)
BCAN	1	HDL-C			2 (0/1/1)
HDGF	1	HDL-C			3 (0/1/2)
PRCC	1	HDL-C			2 (0/2/0)
NTRK1	1	HDL-C			2 (1/1/0)
FBXW7	4	TC, LDL-C, HDL-C	T2D		7 (1/3/3)

The reported genome-wide significant associations to features of metabolic syndrome in genome-wide association studies in human subjects in genes present at genomic regions syntenic to the differential segments of both BN-*Dca* and BN-*Lx*.Dca congenic strains and showing variation between DNA sequences of the SHR and BN-*Lx* parental strains. The presented data are based on the Catalog of Published Genome-Wide Association Studies, available at: http://www.ebi.ac.uk/gwas/ (accessed on Sep 26th, 2016). HSA – human chromosome; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high-density lipoprotein cholesterol; QUICKI – quantitative insulin sensitivity check index; T2D – type 2 diabetes.

CELSR2 – cadherin EGF LAG seven-pass G-type receptor 2; MYBPHL – myosin binding protein H like; SORT1 – sortilin 1; PSMA5 – proteasome subunit α 5; ATXN7L2 – ataxin 7 like 2; GPR61 – G protein-coupled receptor 61; GNAI3 – G protein subunit α i3; CSF1 – colony-stimulating factor 1; AHCYL1 – adenosylhomocysteinase like 1; NOTCH2 – notch 2 (Zeggini et al., 2008); BCL9 – B-cell CLL/lymphoma 9 (Anderson et al., 2015); SETDB1 – SET domain bifurcated 1 (Willer et al., 2013); CERS2 – ceramide synthase 2 (Willer et al., 2013); ANXA9 – annexin A9 (Willer et al., 2013); BNIPL – BCL2 interacting protein like (Willer et al., 2013); CDC42SE1 – CDC42 small effector 1 (Willer et al., 2013); MLLT11 – myeloid/lymphoid or mixed-lineage leukaemia; translocated to, 11 (Willer et al., 2013); GABPB2 – GA binding protein transcription factor β subunit 2 (Willer et al., 2013); PMVK – phosphomevalonate kinase (Willer et al., 2013); DCST2 – DC-STAMP domain containing 2 (Comuzzie et al., 2012); ADAM15 – ADAM metallopeptidase domain 15 (Comuzzie et al., 2012); TRIM46 – tripartite motif containing 46 (Comuzzie et al., 2012); THBS3 – thrombospondin 3 (Comuzzie et al., 2012); BCAN – brevican (Willer et al., 2013); HDGF – hepatoma-derived growth factor (Willer et al., 2013); PRCC – papillary renal cell carcinoma (translocation-associated) (Willer et al., 2013); NTRK1 – neurotrophic receptor tyrosine kinase 1 (Willer et al., 2013); FBXW7 – F-box and WD repeat domain containing 7 (Mahajan et al., 2014).

and BN-*Lx* were reported in syntenic regions of the human genome, some of them showing polymorphisms between the parental strains of the new congenic models (Table 4).

As noted above, several metabolic disturbances have been individually mapped to the region covered by the differential segment of our congenics to date, yet the current study shows a unique combination of decreased lipid levels and increase in insulin, proinflammatory markers triggered by the introgression of a limited genomic region. The effect of the RNO2 differential segment on glucose tolerance is similar to that observed

in the BN-*Lx*.SHR2 strain (Seda et al., 2006). While both new congenic strains displayed distinctively lower values of lipids and higher levels of leptin, the effect of the RNO2 segment was attenuated in the double-congenic BN-*Lx.Dca* for insulinaemia, free fatty acids and pancreatic polypeptide, as well as for several proinflammatory markers.

It has been shown that the amino terminal domain of Cx50 (i.e., the site of the Dca mutation in the new BN-*Dca* and BN-*Lx.Dca* congenic strains) lines the pore of gap junction channels and is essential for the channel conductance and transjunctional voltage-dependent gating as well as in limiting the rate of ion permeation (Xin and Bai, 2013). The connexin subtypes that compose the gap junction channel can affect the pore size of the channel, its switch control for opening and closing (Xin and Bai, 2013). The ability of mutant Cx50 to oligomerize with other connexins to form gap junction channels is well documented (Tong et al., 2011); the resulting functional consequences range from no effect to dominant negative inhibition of the channel function (Pal et al., 1999; Tong et al., 2011).

Our observation of increased insulinaemia in BN-*Dca* is in line with a similar effect in the SHR-*Dca* strain (Seda et al., 2016) and the established importance of gap junction-mediated signalling for the correct function of pancreatic β cells (Head et al., 2012; Cigliola et al., 2016). Using a battery of *in silico* methods, we identified further candidates possibly responsible for the observed metabolic effects. The information is scarce regarding *Ttc24* and *Lingo4* genes predicted to carry mutations impairing the function of the protein product, and neither *Sprr3* nor *Itga10* have so far been linked to conditions related to metabolic syndrome. On the other hand, *Arhgef11* was shown to be a key determinant of mammalian metabolism and obesity-associated pathologies, as its deletion substantially affected susceptibility to diet-induced obesity and type 2 diabetes (Chang et al., 2015) and *MYBPHL* was (together with the neighbouring genes *CELSR2*, *PSRC1*, *MYBPHL*, and *SORT1*) associated with total, low- and high-density cholesterol levels to a wide range of cardiovascular phenotypes, including early onset myocardial infarction, coronary artery calcification, coronary artery stenosis, and abdominal aorta aneurysm in human GWAS (Kjolby et al., 2015).

The effect of the RNO8 differential segment is of PD/Cub origin and only limited information on the genetic variation is available. We have previously identified three non-synonymous amino acid substitutions in *Zbtb16*, *Htr3b*, and *Usp28* genes while studying a limited region of the segment deemed responsible for insulin resistance, dyslipidaemia, hypertension and cardiac fibrosis (Seda et al., 2005; Liška et al., 2014). However, we cannot rule out the possibility of additional genetic variants present in the PD/Cub-derived segment. At this point it is not possible to fully resolve the key gene(s) behind the modulation of metabolic syndrome features in the BN-*Dca* and BN-*Lx.Dca* congenic strains; nevertheless, the highlighted genes (Tables 3 and 4) constitute prime candidates for ensuing investigation. While it can be deduced that variation in gene(s) present within the BN-*Dca* and BN-*Lx.Dca* differential segment is responsible for the observed phenotypic effect, it is also necessary to consider that their action may be nonlinear in nature and that specific perturbed networks including both the variant genes and their interactions with the genomic background should be sought.

The limitations of the current study include the use of only male rats, as sex-specific genetic architecture of the traits defining metabolic syndrome has been described both in man (Seda et al., 2008b) and in experimental models (Ueno et al., 2003; Hoffman et al., 2013). Further studies should address the functional consequences of the identified DNA-level variations, including the *in silico* predicted effects of the non-synonymous mutations in the relevant organ systems and cell types to provide insight into the underlying mechanisms. The prioritization approaches should combine prior art, expression assessment at the RNA and protein levels, narrowing the differential segment and targeted mutagenesis. We have derived two new congenic models that prove that a narrow genomic region of spontaneously hypertensive rat RNO2 carrying, among others, the mutant *Gja8* gene affects several features of metabolic syndrome in a divergent way. By comparing the DNA sequence of the two progenitor strains combined with virtual comparative mapping we were able to identify genetic variants possibly responsible for the observed metabolic effects. This prioritization may considerably accelerate the path towards ascertainment of the causal perturbed networks.

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