Genetic Variation in Renal Expression of *Folate Receptor 1* (*Folr1*) Gene Predisposes Spontaneously Hypertensive Rats to Metabolic Syndrome

Michal Pravenec, Viktor Kožich, Jakub Krijt, Jitka Sokolová, Václav Zídek, Vladimír Landa, Petr Mlejnek, Jan Šilhavý, Miroslava Šimáková, Vojtěch Škop, Jaroslava Trnovská, Ludmila Kazdová, Takashi Kajiya, Jiaming Wang, Theodore W. Kurtz

**Abstract**—Metabolism of homocysteine and other sulfur amino acids is closely associated with metabolism of folates. In this study, we analyzed the possible role of folates and sulfur amino acids in the development of features of the metabolic syndrome in the BXH/HXB recombinant inbred strains derived from the spontaneously hypertensive rat (SHR) and Brown Norway progenitors. We mapped a quantitative trait locus for cysteine concentrations to a region of chromosome 1 that contains a cis-acting expression quantitative trait locus regulating mRNA levels of folate receptor 1 (*Folr1*) in the kidney. Sequence analysis revealed a deletion variant in the *Folr1* promoter region of the SHR. Transfection studies demonstrated that the SHR-promoter region of *Folr1* is less effective in driving luciferase reporter gene expression than the Brown Norway promoter region of *Folr1*. Results in the SHR.BN-chr.1 congeneric strain confirmed that the SHR variant in *Folr1* cosegregates with markedly reduced renal expression of *Folr1* and renal folate reabsorption, decreased serum levels of folate, increased serum levels of cysteine and homocysteine, increased adiposity, ectopic fat accumulation in liver and muscle, reduced muscle insulin sensitivity, and increased blood pressure. Transgenic rescue experiments performed by expressing a *Folr1* transgene in the SHR ameliorated most of the metabolic disturbances. These findings are consistent with the hypothesis that inherited variation in the expression of *Folr1* in the kidney influences the development of the metabolic syndrome and constitutes a previously unrecognized genetic mechanism that may contribute to increased risk for diabetes mellitus and cardiovascular disease. (Hypertension. 2016;67:335-341. DOI: 10.1161/HYPERTENSIONAHA.115.06158.) • Online Data Supplement

**Key Words:** blood pressure • cysteine • folate receptor 1 • metabolic syndrome X • rats, inbred SHR

Metabolic syndrome is a cluster of clinical disturbances (abdominal obesity, hypertension, dyslipidemia, and impaired glucose tolerance/insulin resistance) associated with an increased risk for diabetes mellitus and cardiovascular disease. Mild hyperhomocysteinemia, a common finding in patients with arteriosclerosis, has been described as a possible component of metabolic syndrome.1,2 Because folates and B vitamins modulate metabolism of homocysteine and of other sulfur amino acids (eg, cysteine, methionine, and cystathionine), mild hyperhomocysteinemia may be mediated by nutritional or genetically determined deficiencies of these vitamins. There is a growing body of epidemiological and clinical evidence for an important role of folate deficiency in human metabolic disturbances and hypertension.3–5 We have shown previously that nutritional folate deficiency leads to development of features of the metabolic syndrome in the spontaneously hypertensive rat (SHR),6 a widely studied model of essential hypertension associated with increased risk for insulin resistance and dyslipidemia. In this study, in the BXH/HXB recombinant inbred (RI) strains derived from the SHR strain and the normotensive Brown Norway (BN) strain,7 we used a combination of linkage and correlational analyses of physiological traits and gene expression levels to investigate genetic factors influencing folate and sulfur amino acid metabolism and risk for multiple features of the metabolic syndrome.

**Methods**

**Animals**

SHR/OlaIpcv rats (referred to as the SHR strain), BN-Lv/Cub rats (referred to as the BN strain), BXH/HXB RI strains8 derived from the SHR and BN progenitor strains, SHR.BN-D1Mit51g2 congenic

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rats (referred to as the SHR.BN-chr.1 congenic strain), and SHR/Ola-TgN(EF1aFolr1)Ipcv transgenic rats (referred to as the SHR-Folr1 transgenic strain) were housed in an air-conditioned animal facility and allowed free access to food and water. The SHR.BN-chr.1 congenic strain was derived by backcrossing a 60-Mbp segment of BN chromosome 1 that contains hundreds of genes including folate receptor 1 (Folr1) onto the SHR background. SHR-Folr1 transgenic rats were derived by microinjection of SHR ova with a construct containing Folr1 cDNA of BN origin under control of the EF-1α universal promoter. Biochemical, metabolic, and hemodynamic phenotypes were assessed in 4-month-old nonfasted male rats that were originally raised on standard laboratory chow. Experiments were performed after feeding selected groups of rats either a folate deficient diet for 4 weeks before study (TD 01505 diet with 1% succinylsulfathiazole containing <0.1 mg folate/kg diet, Harlan-Teklad, Madison, WI) or a high fructose diet containing 60% fructose for 2 weeks before study (AB Diets, Woerden, The Netherlands). The low folate diet was used to probe whether transgenic expression of Folr1 in the SHR strain would help protect against folate deficiency. The high fructose diet was used to promote increased susceptibility to genetic risk factors for features of the metabolic syndrome including insulin resistance and dyslipidemia. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic and were approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences, Prague.

Genetic Analysis
Heritability of cysteine and homocysteine levels was estimated by using the variances in scores between and within the RI strains. The additive genetic variance was estimated as 50% of the total variance between the means of the RI strains; the environmental variance was estimated to be the average variance in mean phenotypic values within the RI strains. Narrow heritability was calculated by dividing the additive genetic variance by the sum of the additive genetic variance and the environmental variance. The calculation of heritability corrects for the doubling effects of inbreeding on additive genetic variance by the sum of the additive genetic variance and the environmental variance. The continuously distributed values among the RI strains are consistent with a polygenic mode of inheritance for plasma levels of both homocysteine and cysteine. The heritabilities of plasma homocysteine and cysteine levels were estimated to be 50% and 47%, respectively. Linkage analyses revealed a significant cysteine regulatory QTL on chromosome 1 with the peak of QTL linkage near the D1Arb15 marker in the immediate vicinity of the Folr1 candidate gene (Figure 2A). Plasma levels of total cysteine in the RI strains that inherited the SHR D1Arb15 allele were significantly higher than in those that inherited the BN allele (254±9 versus 210±4 μM; P<0.0001). This QTL is responsible for 50% of the variation in cysteine levels. As discussed further below, plasma levels of homocysteine did not show significant linkage to this QTL region on chromosome 1.

The Folr1 gene found to map near the QTL peak regulating cysteine levels encodes the Folr1 protein that is known to

Parameters of Glucose and Lipid Metabolism and Blood Pressure Measurements
The methods for assessment of skeletal muscle insulin sensitivity, all of the biochemical measurements in serum and tissue, and telemetric blood pressure measurements are described in the online-only Data Supplement.

Statistical Analysis of Metabolic and Physiological Studies and Real Time Polymerase Chain Reaction Data
The methods for statistical analysis are described in the online-only Data Supplement.

Results
Figure 1 shows the distribution of plasma total homocysteine and cysteine concentrations among BXH/HXB RI strains that were fed a high fructose diet for 2 weeks before plasma sampling. The continuously distributed values among the RI strains are consistent with a polygenic mode of inheritance for plasma levels of both homocysteine and cysteine. The heritabilities of plasma homocysteine and cysteine levels were estimated to be 50% and 47%, respectively. Linkage analyses revealed a significant cysteine regulatory QTL on chromosome 1 with the peak of QTL linkage near the D1Arb15 marker in the immediate vicinity of the Folr1 candidate gene (Figure 2A). Plasma levels of total cysteine in the RI strains that inherited the SHR D1Arb15 allele were significantly higher than in those that inherited the BN allele (254±9 versus 210±4 μM; P<0.0001). This QTL is responsible for 50% of the variation in cysteine levels. As discussed further below, plasma levels of homocysteine did not show significant linkage to this QTL region on chromosome 1.

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Folr1 Sequencing and Promoter Analysis
The methods for sequencing and promoter analysis are described in the online-only Data Supplement.

Gene Expression Studies
The methods for gene expression measurement by real time polymerase chain reaction are described in the online-only Data Supplement.
be involved in renal tubular transport of folate.\textsuperscript{14} We found that renal expression level of \textit{Folr1} is strongly controlled by a QTL on chromosome 1 that also maps directly to the physical location of the \textit{Folr1} gene near the QTL peak for cysteine levels (Figure 2A). This \textit{cis}-acting expression QTL on chromosome 1 accounts for almost all of the genetic variation in \textit{Folr1} expression in the kidney. Comparison of the published genome sequences\textsuperscript{15} of SHR and BN rats reveals a 5.7-kb deletion in the \textit{Folr1} promoter region of the SHR strain, which we confirmed by polymerase chain reaction analysis (Figure S1 in the online-only Data Supplement). Transfection studies demonstrated that the SHR-promoter region of \textit{Folr1} is significantly less effective in driving luciferase reporter gene expression than the BN-promoter region of \textit{Folr1} that segregated in the RI strains (Figure 2B). These observations suggest that sequence variation in the promoter region of the \textit{Folr1} gene is responsible for the variation in renal \textit{Folr1} expression observed in the RI strains. In addition, we found significant inverse correlations between renal expression of \textit{Folr1} and circulating levels of homocysteine ($r = -0.51; P=0.005$) and cysteine ($r = -0.41; P=0.02$) across the 30 RI strains further supporting the \textit{Folr1} as a candidate gene for follow-up in vivo functional studies. In Discussion section, we consider why circulating levels of both cysteine and homocysteine correlate with renal expression levels of \textit{Folr1}, whereas only cysteine levels seem to be linked to the \textit{cis}-acting expression QTL for \textit{Folr1} on chromosome 1.

We performed experiments in the SHR.BN-chr.1 congenic strain in which the BN \textit{Folr1} allele on chromosome 1 is expressed on the SHR background under control of its native regulatory elements.\textsuperscript{8} A high fructose diet was used to promote increased susceptibility to the development of features of the metabolic syndrome in these animals. As noted, the SHR.BN-chr.1 congenic strain harbors the \textit{Folr1} gene of the BN strain, which we found contains a more transcriptionally active promoter region than the \textit{Folr1} gene of the SHR.

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**Figure 2.** Linkage mapping and gene expression studies. **A**, Interval mapping of plasma cysteine (solid line) and renal \textit{Folr1} mRNA abundance (dotted line) on chromosome 1 in recombinant inbred (RI) strains. Note the colocalization of the peaks of quantitative trait locus (QTL) linkages for both cysteine levels and renal \textit{Folr1} expression levels. The arrow shows the position of the \textit{Folr1} gene on chromosome 1. Confidence limits of QTL locations are depicted by histograms obtained by a bootstrap procedure (solid bars for cysteine levels and open bars for \textit{Folr1} expression levels). **B**, \textit{Folr1} promoter analysis. Luciferase reporter gene assay showed lower transcriptional activity of the \textit{Folr1} promoter region of the spontaneously hypertensive rat (SHR) strain compared with the Brown Norway (BN) \textit{Folr1} promoter region present in the SHR congenic strain and the RI strains. Regions of $\approx 2000$ base pairs upstream of the \textit{Folr1} gene were tested (Figure S1 for details). *\textit{P}<0.01 compared with SHR. ** Renal expression of the \textit{Folr1} gene in the SHR strain (open bars) was significantly lower when compared with the SHR.BN-chr.1 congenic strain (solid bars). All rats in these experiments were fed a high fructose diet. This difference in \textit{Folr1} gene expression was apparent in studies of the long-form \textit{Folr1} transcript and studies of the both short- and long-form \textit{Folr1} transcripts together (different length transcripts caused by alternative splicing). The results are expressed as fold changes relative to the mean expression level in the SHR controls of the short and long transcripts combined that was arbitrarily defined as 1. * Significant difference of \textit{P}<0.05 between the SHR and SHR congenic strain with respect to expression of the long-form transcript and ** significant difference of \textit{P}<0.01 between the SHR and SHR congenic strain with respect to expression of both short and long transcripts combined. **D**, The expression of the \textit{Folr1} transgene in various organs (depicted by the grey bars). Renal expression of \textit{Folr1} was significantly greater in the transgenic strain than in the SHR strain. The transgenic strain also expressed \textit{Folr1} in liver, fat, and soleus muscle where little or no \textit{Folr1} is ordinarily expressed. Congenic rats in these experiments were fed a high fructose diet and transgenic rats a folate deficient diet. *\textit{P}<0.01 and **\textit{P}<0.001 compared with SHR strain. **\textit{P}<0.0001 compared with SHR and SHR congenic strain where no \textit{Folr1} was detected.
Metabolic studies revealed significantly lower urinary folate excretion and fractional folate excretion in the congenic strain than in the SHR progenitor strain (Table, high fructose diet). These data indicate enhanced renal folate reabsorption in the congenic strain, which is also accompanied by significantly higher plasma levels of folate, methionine and serine and significantly lower levels of plasma total homocysteine and total cysteine (Table, high fructose diet). In addition, congenic rats showed lower body weight because of reduced adiposity as suggested by 17% reduction of epididymal fat weight and lower ectopic accumulation of triglycerides in liver and muscle (Table, high fructose diet). The reduced accumulation of triglycerides was associated with amelioration of insulin resistance in skeletal muscle as shown by 2.6- and 2.3-fold increases in basal and insulin stimulated glycogenesis, respectively (Table, high fructose diet). SHR.BN-chr.1 congenic rats exhibited reduced blood pressure when compared with the SHR as was published previously.8

Transgenic rats, derived by microinjections of SHR ova with the construct containing BN Folr1 cDNA under control of EF-1α universal promoter, exhibited renal expression of the Folr1 that was fairly comparable with the expression level of endogenous Folr1 observed in the congenic strain and significantly higher than the renal expression level of endogenous Folr1 in the SHR progenitor strain (Figure 2D). In the transgenic strain, expression of Folr1 was also observed in various tissues where Folr1 is ordinarily not well expressed (eg, liver, fat, soleus muscle; Figure 2D). Thus, the following metabolic studies in the transgenic strain should be interpreted with caution and be considered in context with the studies in RI strains and the congenic strain in which the Folr1 gene is expressed under control of its native regulatory elements.

To test whether transgenic expression of Folr1 would protect against the effects of dietary folate restriction on folate status and metabolic disturbances in the SHR model, we studied the SHR progenitor rats and Folr1 transgenic rats after feeding

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SHR indicates spontaneously hypertensive rat; BN, Brown Norway; and Folr1, folate receptor 1.

*Rats from the SHR strain (N=10) and the SHR.BN-D1Mit3/Igf2 (Folr1) congenic (N=8) strain. Rats were fed standard laboratory chow from weaning and then fed a high fructose diet for 2 weeks before end of the study and blood sampling.

†Rats from the SHR (N=9) and SHR-Folr1 transgenic (N=11) strain. Rats were fed a folate deficient diet for 4 weeks before the metabolic studies.

‡P<0.05 versus SHR control.

§P<0.001 versus SHR control.
a folate deficient diet for 4 weeks. As expected, administration of the folate deficient diet caused extremely low steady state levels of urinary excretion of folate and fractional excretion of folate in both groups (Table, folate deficient diet). However, plasma and red cell folate levels were significantly higher in the Folr1 transgenic strain than in the SHR progenitor strain suggesting that the transgenic strain may have achieved the low steady state level of folate excretion more rapidly and conserved folate more efficiently than the SHR progenitor strain. In addition, transgenic rats compared with SHR exhibited lower homocysteine and cysteine levels, lower relative epididymal fat pad weight and liver weight, reduced serum triglycerides, and 3.1-fold and 1.5-fold lower levels of ectopic triglyceride accumulation in liver and in muscle, respectively (Table, folate deficient diet). Moreover, expression of the Folr1 transgene attenuated impairment in insulin resistance and disturbances in glucose and insulin levels observed in rats fed the folate deficient diet (Table, folate deficient diet). No differences in blood pressure levels were detected between transgenic rats and controls when both groups were fed either a normal diet or a folate deficiency diet (data not shown).

To search for possible molecular mechanisms that would explain the role of folate deficiency and elevated cysteine levels in increased adiposity, ectopic fat accumulation, and insulin resistance observed in the SHR strain compared with the Folr1 transgenic strain and congenic strain, we determined expression of 2 genes that have been implicated in the pathogenesis of obesity because of increased cysteine levels, namely, stearyl-CoA desaturase-1 (Scd1) and peroxisome proliferator-activated receptor gamma (Pparg), which is known to encode a transcription factor that regulates the expression of Scd1. As shown in Figure 3, the expression levels of Scd1 and Pparg in liver and adipose tissue were significantly greater in the SHR progenitor strain than in the SHR-Folr1 transgenic strain, which ectopically expresses Folr1 in these tissues. However, the expression levels of these genes were also greater in liver and fat tissue of the SHR compared with that in the SHR congenic strain, which does not ectopically express Folr1 in these tissues.

**Discussion**

In these current studies in the SHR model, we investigated whether risk for features of the metabolic syndrome might be influenced by genetically determined variation in folate and sulfur amino acid metabolism. We found that genetically determined reductions in expression levels of Folr1 may be contributing to decreased renal tubular reabsorption of folate, reduced plasma folate levels, increased plasma levels of cysteine, and consequent disturbances in lipid and glucose metabolism. We implicate a functional role for a promoter variant in Folr1 in these processes based on a combination of genetic and metabolic studies in RI, transgenic, and congenic strains together with promoter function studies in HEK293 cells.

Mechanisms that may connect folate deficiency with disturbances of lipid and glucose metabolism are not fully understood. One possibility is that reduced availability of folate for homocysteine remethylation might result in deficiency of S-adenosylmethionine and accumulation of S-adenosylhomocysteine, which together may reduce production of phosphatidylcholine, an essential factor for very-low-density lipoprotein assembly and transport of triglycerides out of the liver. To explore this possibility, further experiments are needed to measure phosphatidylcholine production in the SHR progenitor strain and SHR-Folr1 transgenic and congenic strains and to test for a possible association of reduced phosphatidylcholine availability with ectopic fat accumulation in tissues.

It has recently been proposed that increased availability of cysteine may promote obesity, and thus, increased cysteine levels could represent another mechanism that might mediate effects of folate deficiency and impaired homocysteine remethylation on risk for features of the metabolic syndrome. Impaired homocysteine remethylation related to a reduction in folate availability could increase flux of metabolites through the transsulfuration pathway thereby contributing to the greater cysteine levels observed in the SHR. In the SHR compared with the Folr1 congenic and transgenic strains, increased plasma concentrations of cysteine and glycine, and decreased plasma concentrations of methionine and serine, would be consistent with a decreased rate of homocysteine remethylation due to relative deficiency of folate. Recent studies suggest that alterations in cysteine levels may influence risk for obesity through effects on expression of Scd1, which may be mediated in part via transcriptional effects of PPARG.
disturbances via effects on Scd1. Consistent with this possibility, we found that in the Folr1 congenic and transgenic strains compared with the SHR progenitor strain, lower cysteine levels and lower expression levels of Pparg and Scd1 were associated with improvements in glucose metabolism and in lower levels of adiposity and ectopic fat accumulation. The greater ectopic fat accumulation associated with relative folate deficiency in the SHR is in good agreement with previous reports demonstrating that dietary folate deficiency induces hepatic steatosis.

Although we observed that renal expression levels of Folr1 correlated with circulating levels of both cysteine and homocysteine, the Folr1 expression levels and cysteine levels were linked to the Folr1 promoter variant but homocysteine levels were not. What could be the explanation for this? Homocysteine is at the intersection among transmethylation, transsulfuration, and remethylation, and many factors can affect homocysteine levels. Thus, it is possible that more genetic factors are involved in increasing homocysteine levels than cysteine levels. In addition, it is possible that homocysteine levels may influence Folr1 expression, and that some of the correlation between Folr1 expression levels and homocysteine levels may involve a reverse causation effect.

In this study, transgenic augmentation of Folr1 expression levels in the SHR did not affect blood pressure. This observation suggests that reduced Folr1 expression levels are not contributing to the hypertension in the SHR strain. In previous studies of the SHR and SHR.BN-chr.1 congenic strain, we found lower blood pressure in the SHR.BN-chr.1 congenic strain, which has greater Folr1 expression levels than the SHR. However, the differential chromosome segment in the SHR.BN-chr.1 congenic strain contains many other genes besides just Folr1 that might affect folate transport and blood pressure. Specifically, it should be noted that the Folh1 gene encoding folate hydrolase 1, which could affect cellular transport of folate in intestine and possibly other tissues is closely linked to Folr1 on rat chromosome 1 within the differential chromosome segment of the SHR.BN-chr.1 congenic strain. Although the role of folate hydrolase 1 in folate transport in the rat intestine is controversial, the results of recent studies in Folh1 knockout mice suggest that genetic deficiency of Folh1 increases blood pressure. Thus, further studies will be of interest to determine whether genetic variation in Folh1 is contributing to effects on folate metabolism and blood pressure in the SHR compared with the SHR.BN-chr.1 congenic strain. Knockout of Folr1 in the mouse is embryonically lethal, and the effects of genetic variation in Folr1 expression on blood pressure and metabolic traits in mice remain to be determined. Partially rescued preterm Folr1−/− fetuses were found to have outflow tract defects, aortic arch abnormalities, and isolated dextrocardia. To the best of our knowledge, no metabolic traits were studied in these mice.

Although the exact relevance of folate metabolism in the SHR model to pathogenesis of metabolic syndrome in humans requires additional study, several lines of evidence strongly suggest that folate deficiency plays a role in at least some features of metabolic syndrome in humans. In addition, administration of folic acid or 5-methyltetrahydrofolate has been reported to improve endothelial dysfunction, decrease blood pressure, and ameliorate insulin resistance. Recent reports suggest that MTHFR c.677C>T homozygous individuals with genetically determined decreases in folate levels exhibit features of metabolic syndrome, namely low birth weight and higher insulin levels, and that folate supplementation in pregnant women has the transgenerational effect of reducing insulin resistance in offspring. These observations point to a possible role of folate metabolism in the pathogenesis of metabolic syndrome in humans and should motivate further studies on this topic. FOLR1 deficiency in humans is associated with abnormal transport of methyltetrahydrofolate across the blood–brain barrier and with neurocognitive impairment. In the GWAS (Genome Wide Association Studies) database at www.gwascentral.org, we have not found any reports of an association of the FOLR1 locus with cardiometabolic traits. However, folate intake was not monitored in all GWAS studies and depending on folate intake, there might be subsets of people in whom human FOLR1 variants are affecting metabolic phenotypes.

In summary, the results of linkage analysis, gene expression and sequencing studies, and in vivo functional experiments suggest that in the SHR model, genetically determined reductions in renal expression of Folr1 may be contributing to decreased renal tubular reabsorption of folate, reduced plasma folate levels, increased plasma levels of cysteine, and consequent disturbances in lipid and glucose metabolism. Thus, variants in Folr1 may be contributing to genetic risk for features of the metabolic syndrome in the SHR strain. These findings raise the possibility that a previously unrecognized genetic mechanism involving effects on Folr1 expression may contribute to the pathogenesis of common metabolic disturbances associated with increased risk for diabetes mellitus and cardiovascular disease.

Perspectives

The results of these studies are consistent with the hypothesis that folate deficiency related to genetic variation of Folr1 expression in the kidney may predispose to increases in cysteine levels and risk for several features of the metabolic syndrome in the SHR model. These findings together with human studies pointing to a possible role of folates and cysteine in the pathogenesis of insulin resistance, hypertension, and abdominal obesity should motivate further studies on the role of variants in Folr1 and related genes in the risk for metabolic syndrome, diabetes mellitus, and cardiovascular disease.

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Disclosures
None.

References

What Is New?
- These studies indicate that genetically determined variation in activity of a renal folate transporter seems to influence risk for folate deficiency, high cysteine levels, and features of the metabolic syndrome in spontaneously hypertensive rats.

What Is Relevant?
- These findings are consistent with studies suggesting that folate deficiency and increased levels of cysteine may influence development of obesity and related features of the metabolic syndrome in humans.

Novelty and Significance

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Summary

The current results raise the possibility that genetically determined variation in folate transport in the kidney may influence the pathogenesis of common metabolic disturbances associated with increased risk for diabetes mellitus and cardiovascular disease.

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ONLINE DATA SUPPLEMENT

Genetic Variation in Renal Expression of Folr1 (folate receptor 1) Gene Predisposes Spontaneously Hypertensive Rats to Metabolic Syndrome

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Short title: Folr1 and metabolic disturbances in the SHR

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**Supplementary Methods**

**Biochemical parameters.** Folate levels in serum, erythrocytes and urine were determined by the Folate III Assay Kit (Roche GmbH, Basel, Switzerland) (the coefficient of variation for the assays for folate is <5%).\(^1\) Concentrations of total homocysteine and cysteine in plasma were determined by reversed-phase HPLC with fluorescent detection after derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F). The reduction of disulfides and protein bound homocysteine and cysteine was performed with tris(2-carboxyethyl)phosphine (TCEP) as described previously (the coefficients of variation for the assays for homocysteine and cysteine are <3%).\(^2\)

Amino acids in plasma (glycine, serine, cystathionine, methionine, sarcosine) were determined by LC-MS/MS using commercially available kit for amino acid analysis (EZ:faast, Phenomenex, Torrance, USA). Sample preparation according to the manufacturer's instruction involved a solid phase extraction step, derivatisation with propyl chloroformate, and an extraction into an organic solvent prior to LC-MS/MS analysis. The internal standard solution, which is a component of the kit, was supplemented with 1 μmol/L cystathionine-d₄ and sarcosine-d₃ (CDN Isotopes, Canada) and 100 μmol/L of serine-d₃ (Cambridge Isotope Laboratories Inc., USA) and glycine-d₃ (Sigma-Aldrich, Czech Republic).

Blood glucose levels were measured by the glucose oxidase assay (Erba-Lachema, Brno, Czech Republic) using tail vein blood drawn into 5% trichloracetic acid and promptly centrifuged. Serum triglyceride concentrations were measured by standard enzymatic methods (Erba-Lachema, Brno, Czech Republic). Serum insulin concentrations were determined using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden) (the coefficient of variation for the assays for insulin is <3%).

**Tissue triglyceride measurements.** For determination of triglycerides in liver and soleus muscle, tissues were powdered under liquid N₂ and extracted for 16 hours in chloroform:methanol, after which 2% KH₂PO₄ was added and the solution was centrifuged. The organic phase was removed and evaporated under N₂. The resulting pellet was dissolved in isopropyl alcohol and triglyceride content was determined by enzymatic assay (Erba-Lachema, Brno, Czech Republic).

**Basal and insulin stimulated glycogen synthesis in skeletal muscle.** For measurement of insulin stimulated incorporation of glucose into glycogen, diaphragmatic muscles were incubated for 2 hours in 95% O₂ + 5% CO₂ in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.1 μCi/ml of \(^{14}\)C-U glucose, 5 mmol/L of unlabeled glucose, and 2.5 mg/ml of bovine serum albumin (Fraction V, Sigma, Czech Republic), with or without 250 μU/ml insulin. Glycogen was extracted, and insulin stimulated incorporation of glucose into glycogen was determined.

**Blood pressure measurement.** Arterial blood pressures were measured continuously by radiotelemetry (Data Sciences International, St. Paul, U.S.A.) in paired experiments between conscious, unrestrained male rats. All rats were allowed to recover for at least 7 days after surgical implantation of radiotelemetry transducers before the start of blood pressure recordings. Pulsatile pressures were recorded in 5-second bursts every 10 minutes throughout the day and night, and 24-hour averages for systolic and diastolic arterial blood pressure were
calculated for each rat. The results from each rat in the same group were then averaged to obtain the group means for the study.

**Folr1 sequencing and promoter analysis.** DNA sequencing was performed on PCR amplified products using an Applied Biosystems 3730xl DNA Analyzer and the BigDye Terminator v 3.1 Cycle sequencing kit (Applied Biosystems). The GenBank submission IDs are KU095825, KU095826 and KU095827. CR primers are described in the online data supplement as shown in Supplementary Figure 1. Luciferase reporter gene assays were used to compare transcriptional activity of the Folr1 promoter region of the SHR strain to that of the Folr1 promoter region of the BN strain. Transfection studies were performed in HEK293 cells cultured in 10% fetal bovine serum. We cotransfected the cells with a firefly luciferase reporter vector pGL3 including approximately 2,000 base pairs in the region immediately upstream of the Folr1 coding sequences of the SHR and BN strains, together with a renilla luciferase reporter vector under control of the CMV promoter as a reference plasmid. The transfected sequences are depicted in Supplementary Figure 1. Both promoter constructs were directly verified by DNA sequencing. Firefly luciferase activity was normalized by renilla luciferase activity and the results (n=3 in each group) expressed as fold change relative to that of results with the empty pGL3 control vector.

**Gene expression.** Real-time PCR analysis was used to determine expression levels of endogenous Folr (short and long form transcripts), transgenic Folr1, endogenous Pparg, and endogenous Scd1. Total RNA was isolated using standard methods and cDNA was prepared and analyzed by real-time PCR testing using QuantiTect SYBR Green reagents (Qiagen, Inc, Valencia, U.S.A.) on an Opticon continuous fluorescence detector (MJ Research, Waltham, U.S.A.). For all real time PCR studies in which gene expression levels were compared between strains within a given tissue, the gene expression levels were normalized in relation to expression of an internal housekeeping gene encoding Ppia (peptidylprolyl isomerase A, also known as cyclophilin). These expression results are reported as fold increases in relation to the mean expression level in the SHR strain that was arbitrarily defined as 1. For studies comparing renal expression of Folr1, the results are reported as fold increases in relation to the mean total expression level of both short and long form transcripts combined in the SHR strain. For tissues in which the expression levels of Folr1 were undetectable in the SHR strain, the results are reported without referencing them to the expression values in the SHR strain (which was zero). The following primer pair was used to determine expression of total Folr1 mRNA (short and long forms combined). This primer pair also detects expression of the Folr1 transgene: 5'-gcc cag agg aca aat tac a; 5'-cca gtt gac tcg gta cag g. To detect expression of the long form transcript of Folr1, the following primer pair was used: 5'-cgc tga tct gga agc ata a; 5'-ttg gtc cct gag gag aag t. Expression levels of Scd1 and Pparg genes were determined with the following primer pairs: Scd1 upstream primer 5'-tct cgt gga cct cct cct cct cct gga cct cca cct gag cct cca c and downstream primer 5'-tct tct gga cct cct cct cgc gag gat ac; Pparg upstream primer 5'- cca gga gag gct cta cag cca c and downstream primer 5'-aaa ttt ctt agg tgt cag a. The primer pair for detection of cyclophilin was: upstream 5'- agg ata cag gtc cta gca cct gca gca cct cca gca c; downstream, 5'- tca cct tcc caa aga cca c. Statistical analysis of gene expression data is described in the methods section on genetic and statistical analysis.

**Statistical analysis of metabolic and physiologic studies and real time PCR.** Summary results are expressed as means ± SEM. Analysis of gene expression data was performed using the Relative Expression Software Tool (version REST 2009) that tests for significant differences by a randomization procedure. For the analysis of transgene expression in tissues where endogenous Folr1 is not expressed, the results were analyzed in a one sample signed
rank test with the hypothesized sample median = 0. For other data, ANOVA or Kruskal Wallis testing was used for comparisons across three groups with subgroup comparisons made against the SHR strain as the control with adjustments for multiple comparison. Comparisons involving only two groups were performed by unpaired Student t-test or a rank sum test with statistical significance defined as P<0.05 for the two group comparisons.

References

Supplementary Figure S1. Sequence of *Folr1* present in both the Brown Norway (BN) strain and the SHR congenic strain that harbors a segment of chromosome 1 from the BN strain including *Folr1*. In the current sequence analysis of *Folr1*, we have resolved indeterminate nucleotide stretches present in the published genome assemblies of the BN strain. The region of *Folr1* that is deleted in the SHR progenitor strain is shown in upper case bold font.

The deletion in the SHR strain was confirmed by amplifying and sequencing the region that is located between, and includes, the following two sequences that are in upper case font and
underlined by dashed lines: CCTGGGGCTGATTATTAACTGG and GAAGGGGTCAGAGTGAACATGAC. A PCR gel confirming the SHR deletion between and including these two sequences is shown at the end of the sequencing results.

The region of the BN strain that was tested in the promoter function studies is located between, and includes, the following two sequences that are in upper case font and underlined by solid lines: TGTCTGCTGGAGATCACAGG (actual primer) and TCTAATCATGTTTCCTTTCCGG (actual primer CCGAAAGGACCATGATTAGA).

The region of the SHR strain that was tested in the promoter function studies is located between, and includes, the following two sequences that are in upper case font and underlined by solid lines: TGTCTGCTGGAGATCACAGG (actual primer) and CTGCAAAACAGGAAGAGGGAG (actual primer CTCCCTCTCTCTGTTTGCAG).

Exon 1 included in the long form transcript is shown in upper case font shaded in grey. Exon 1 is not included in the short form transcript. Exon 2 included in the short form transcript is shown in lower case font shaded in grey. Exon 2 is the beginning of the translated region. The other exons are shown in lower case font without shading or underlining. Untranslated regions are shown in underlined lower case font.

Gel photo confirming SHR deletion as indicated by the smaller PCR fragment obtained with the PCR amplification of SHR DNA.