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RAPID COMMUNICATION

Dissecting the Role of *Folr1* and *Folh1* Genes in the Pathogenesis of Metabolic Syndrome in Spontaneously Hypertensive Rats

J. ŠILHAVÝ[1], J. KRIJT[2], J. SOKOLOVÁ[2], V. ZÍDEK[1], P. MLEJNEK[1], M. ŠIMÁKOVÁ[1], V. ŠKOP[3], J. TRNOVSKÁ[3], O. OLIYARNYK[3], I. MARKOVÁ[3], M. HÜTTL[3], H. MALÍNSKÁ[3], L. KAZDOVÁ[3], F. LIŠKA[4], V. KOŽICH[2], M. PRAVENEC[1]

[1]Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic, [2]Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic, [3]Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [4]Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic

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Summary

Increased levels of plasma cysteine predispose to obesity and metabolic disturbances. Our recent genetic analyses in spontaneously hypertensive rats (SHR) revealed mutated *Folr1* (folate receptor 1) on chromosome 1 as a quantitative trait gene associated with reduced folate levels, hypercysteinemia and metabolic disturbances. The *Folr1* gene is closely linked to the *Folh1* (folate hydrolase 1) gene which codes for an enzyme involved in the hydrolysis of dietary polyglutamyl folates in the intestine. In the current study, we obtained evidence that *Folh1* mRNA of the BN (Brown Norway) origin is weakly but significantly expressed in the small intestine. Next we analyzed the effects of the *Folh1* alleles on folate and sulfur amino acid levels and consecutively on glucose and lipid metabolism using SHR-1 congenic sublines harboring either *Folr1* BN and *Folh1* SHR alleles or *Folr1* SHR and *Folh1* BN alleles. Both congenic sublines when compared to SHR controls, exhibited significantly reduced folate clearance and lower plasma cysteine and homocysteine levels which was associated with significantly decreased serum glucose and insulin concentrations and reduced adiposity. These results strongly suggest that, in addition to *Folr1*, the *Folh1* gene also plays an important role in folate and sulfur amino acid levels and affects glucose and lipid metabolism in the rat.

Key words

Spontaneously hypertensive rat • *Folr1* gene • *Folh1* gene • Folate • Cysteine • Metabolic syndrome • Folate hydrolase • Glutamate carboxypeptidase

Corresponding author

M. Pravenec, Institute of Physiology of the Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czech Republic. E-mail: pravenec@biomed.cas.cz

Introduction

Increased levels of plasma cysteine predispose to obesity and metabolic disturbances (Carter and Morton 2016, Elshorbagy *et al.* 2012). Since folate and B vitamins modulate metabolism of sulfur amino acids including cysteine, mild hypercysteinemia may be a secondary consequence of deficiencies of these vitamins (Shane 2010). Our recent linkage, congenic and transgenic rescue experiments in spontaneously hypertensive rats (SHR) revealed a mutated *Folr1* (folate receptor 1) as a quantitative trait gene (QTG) when genetically determined reduced renal expression of *Folr1* was associated with decreased renal folate reabsorption, lower folate levels, hypercysteinemia and metabolic disturbances (Pravenec *et al*. 2016). The *Folr1* gene

(position on chromosome 1 at 166 Mbp) is closely linked to another candidate gene, *Folh1* (folate hydrolase 1) which is also known as glutamate carboxypeptidase II (GCPII) (position on chromosome 1 at 150 Mbp). Dietary folates are composed of a mixture of monoglutamyl and polyglutamyl forms that are hydrolyzed to the monoglutamyl form prior to transport across the jejunal brush border membrane (Shane 2010). The *Folh1* gene codes for an enzyme that is predominantly involved in the hydrolysis of dietary polyglutamyl folates by sequential cleaving terminal γ-linked glutamate residues from dietary polyglutamyl folates. However, it has been reported that in Sprague-Dawley rats *Folh1* is not expressed in the small intestine and dietary folates are hydrolyzed by pancreatic γ-glutamyl hydrolase (*Ggh*) (Shafizadeh and Halsted 2007). In the current study, we tested the hypothesis that *Folh1* allele of unrelated BN (Brown Norway) strain, which was derived from wild rats, is expressed in the small intestine and analyzed its effects on folate and sulfur amino acid levels and on parameters of lipid and glucose metabolism using SHR-1 congenic sublines. Our results provided evidence that both reduced renal folate reabsorption due to downregulated *Folr1* renal expression and possibly reduced intestinal folate absorption due to downregulated or practically nonexistent intestinal *Folh1* expression predispose the SHR to relative folate deficiency, hypercysteinemia and disturbances of glucose and lipid metabolism.

Materials and Methods

Animals

SHR/OlaIpcv rats (referred to as the SHR strain), SHR.BN-*D1Rat272*/*Igf2* congenic strain (referred to as the SHR-1 congenic strain) (St Lezin *et al*. 1997), and SHR-1 sublines that harbor either *Folr1* allele of BN origin and *Folh1* allele of SHR origin (referred to as SHR.BN-*Folr1* subline) or *Folr1* allele of SHR origin and *Folh1* allele of BN origin (referred to as SHR.BN-*Folh1* subline), and the BXH/HXB recombinant inbred (RI) strains (Hübner *et al*. 2005) were housed in an air-conditioned animal facility and allowed free access to standard food (Altromin 1314 diet, Lage, Germany) and water. The SHR.BN-*Folr1* and SHR.BN-*Folh1* sublines were selected from (SHR x SHR-1)F2 rats (N=207) with the following primers that distinguished the SHR and BN *Folr1* and *Folh1* alleles. For the *Folr1* gene promoter: F primer CCA CCA TAC CTT GGA GCA GT, R primer CCC AAA TTC CAA ACA ACC TG; for the *Folh1* gene intron: F primer ATG TGTGCG TGC GTA TTC AG, R primer TAG CTG CTG ACT TTG TTG G. The animals with appropriate recombinations were backcrossed to SHR strain and then heterozygotes were intercrossed and differential chromosome segments in both sublines were fixed and homozygous rats were used for phenotyping. Biochemical and metabolic phenotypes were assessed in 4 month old nonfasted male rats (N=8 per group). Tissues for biochemical analyses were collected from non-fasted rats between 9 and 10 AM. 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 of the Czech Academy of Sciences, Prague.

Biochemical parameters

Folate levels in serum 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 %). Concentrations of total homocysteine, cysteine, glutathione (GSH), GSH precursor gamma-glutamylcysteine and GSH degradation product cysteineylglycine in plasma were determined by reversed-phase HPLC with fluorescent detection after derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. The reduction of disulfides and protein bound homocysteine and cysteine was performed with tris(2-carboxyethyl)phosphine as described previously (the coefficients of variation for the assays for homocysteine and cysteine are <3 %).

Blood glucose levels were measured by the glucose oxidase assay (Erba-Lachema, Brno, Czech Republic) using tail vein blood drawn into 5 % trichloroacetic acid and promptly centrifuged. NEFA levels were determined using an acyl-CoA oxidase-based colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany). 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).

Tissue triglyceride measurements

For determination of triglyceride concentrations in liver and soleus muscle, tissues were powdered under liquid N_2 and extracted for 16 h in chloroform:methanol, after which 2 % KH_2PO_4 was added and the solution was

centrifuged. The organic phase was removed and evaporated under N_2. 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 h in 95 % O_2 + 5 % CO_2 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.

Parameters of oxidative stress

Oxidative stress was measured according to the activities of anti-oxidative enzymes, concentrations of glutathione, the major physiological mechanism response to oxidative stress and concentrations of lipoperoxidation products. The activity of superoxide dismutase (SOD) was analyzed using the reaction of blocking nitrotetrazolium blue reduction and nitroformazan formation. Catalase (CAT) activity measurement was based on the ability of H_2O_2 and ammonium molybdate to combine to produce a color complex detected spectrophotometrically. The activity of seleno-dependent glutathione peroxidase (GSH-Px) was monitored by oxidation of gluthathione using Ellman's reagent (0.01 M solution of 5,5'-dithiobis-2 nitrobenzoic acid). The concentration of reduced glutathione (GSH) was determined by the reaction of SH-groups using Ellman's reagent. Glutathione reductase (GR) activity was measured by the decrease of absorbance at 340 nm using a millimolar extinction coefficient of 6220 $M^{-1}cm^{-1}$ for NADPH (using the Sigma assay kit). Lipoperoxidation products were assessed according to concentrations of thiobarbituric acid-reactive substances (TBARS) determined by assaying the reaction with thiobarbituric acid. Concentrations of conjugated dienes were analyzed by extraction in the media (heptan:isopropanol = 2:1) and measured spectrophotometric in heptan's layer.

Folh1 sequencing

cDNA 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, Waltham, USA). The PCR primers were: *Folh1*-6F 5'-TGC AGA CTC TCT GCA GTA GA-3' and *Folh1*-3007R 5'-GAA GAT AAC AAT GAA AAA TAG AAA-3'.

Western blotting

Tissues were homogenized in aqueous buffer (50 mM Tris pH 8, 120 mM NaCl and 0.5 % NP-40) supplemented by protease inhibitor coctail Complete (Roche, Basel, Switzerland) using TissueLyser (Qiagen, Hilden, Germany). Lysates were run on SDS-PAGE (10 % separating gel), proteins were blotted onto PVDF membranes Immobilon P (EMD Millipore Biosciences, Billerica, Massachusetts, USA). Membranes were incubated overnight at 4 °C with anti-GCPII (FOLH1) mouse monoclonal antibody (generous gift of Pavel Šácha and Jan Konvalinka, described by Rovenská *et al.* 2008) at final dilution 1:5,000. Secondary HRP-conjugated antibody was from GE Healthcare Bio-Sciences (Little Chalfont, UK), and signal was detected using ECL Prime chemiluminiscent detection kit (GE Healthcare Bio-Sciences, Pasching, Austria) and Hyperfilm ECL.

Gene expression

Real-time PCR analysis was used to determine expression levels of *Folh1* gene in the intestine. 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, USA) on an Opticon continuous fluorescence detector (MJ Research, Waltham, USA). 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). The primer pair for detection *Folh1* was: *Folh1*-1526F TGA AGG CTT TGA AGG CAA AT and *Folh1*-1670R GCC TGA AGC AAT TCC AAG TC. The primer pair for detection of cyclophilin was: F primer 5′-AGC ATA CAG GTC CTG GCA T; R primer 5′-TCA CCT TCC CAA AGA CCA C.

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 (Pfaffl *et al.* 2002). Bichemical and metabolic data were analyzed by One-way ANOVA with Holm Sidak testing for comparisons across three groups with subgroup comparisons made against the SHR strain as the control with adjustments for multiple comparisons.

Results

Linkage, sequence, and expression analyses

We have genotyped the RI strains using primers that distinguished SHR and BN *Folh1* alleles and found that the strain distribution pattern of *Folh1* alleles (at position 150 Mbp) is identical to that of the *D1Arb15* marker (at position 154 Mbp) that was previously located at the peak of the QTL linkage for serum cysteine (Pravenec *et al.* 2016). Thus *Folh1* gene itself is located at the peak of QTL linkage for plasma cysteine levels. Sequence analysis of the SHR *Folh1* allele when compared to BN sequence revealed 2 amino acid substitutions (BN→SHR) p.Arg15Gly and p.Thr545Asn. Compared to SHR rats that practically did not express *Folh1*, SHR-1 congenic rats with the *Folh1* allele of the BN origin exhibited weak but significant expression of *Folh1* in the small intestine (Fig. 1). Western blot analysis revealed low expression of FOLH1 protein in the intestines isolated from both SHR and SHR-1 congenic rats but there was no significant difference between the strains (data not shown).

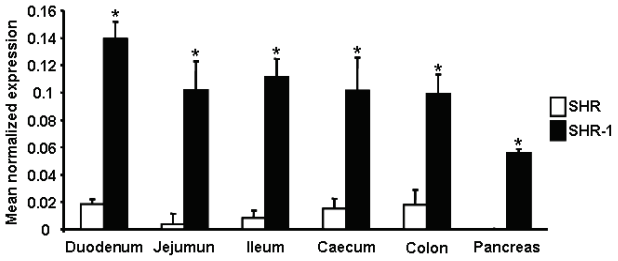

Fig. 1. The expression of the *Folh1* gene in the intestine. Real time PCR revealed significantly reduced expression of *Folh1* mRNA in the intestine of the SHR when compared to the SHR-1 congenic strain. * denotes P<0.01.

Table 1. Metabolic phenotypes in SHR versus SHR.BN-*Folr1* and SHR.BN-*Folh1* congenic sublines.

Traits	SHR	SHR.BN-*Folr1* subline	SHR.BN-*Folh1* subline
Serum folate (nmol/l)	37.8±1	37±1	36±2.1
Urinary excretion of folate (μg/min)	4.7E-04±9E-05	2.6E-04±2E-05*	3E-04±4E-05
Folate clearance (ml/kg/min)	0.047±0.007	0.023±0.002*	0.028±0.004*
Plasma total homocysteine (μmol/l)	4.3±0.4	3.4±0.5*	3.3±0.3*
Plasma total cysteine (μmol/l)	230±16	188±26*	194±23*
Plasma cysteinyl-glycine (μmol/l)	3.3±0.3	3±0.5	2.9±0.3*
Plasma glutathione (μmol/l)	58±12	61±9	57±4
Plasma γ-glutamylcysteine (μmol/l)	6.5±0.5	4.8±0.6*	5.1±1.1*
Body mass (g)	320±7	310±7	304±7
Epididymal fat mass (g/100g BW)	1.14±0.04	1±0.07*	0.96±0.33*
Serum glucose (mmol/l)	7.8±0.3	7±0.2*	6.7±0.1*
Serum insulin (nmol/l)	0.45±0.03	0.28±0.03*	0.27±0.03*
Serum triglycerides (mmol/l)	2.8±0.2	3.1±0.3	2.9±0.3
Serum NEFA (mmol/l)	0.77 ±0.08	0.9±0.07	1.11±0.05*
Liver triglycerides (μmol/g)	14.1±0.8	14.4±0.9	13.1±0.6
Muscle triglycerides (μmol/g)	3.6±0.9	5.5±0.6	3.7±0.3
Basal glycogenesis (nmol glucose/g/2 h)	80±11	100±11	75±20
Insulin stimulated glycogenesis (nmol glucose/g/2 h)	201±25	216±27	199±35

* denotes P<0.05 versus SHR control.

Table 2. Parameters of oxidative stress in the SHR and SHR.BN-*Folr1* and SHR.BN-*Folh1* congenic sublines.

Trait	SHR	SHR.BN-*Folr1*	SHR.BN-*Folh1*
Liver			
SOD (U/mg)	0.161±0.016	0.129±0.008*	0.118±0.005*
GSH-Px (μMGSH/min/mg)	228±14	199±19	151±10*
GR (μM NADPH/min/mg)	108±6	122±10	111±8
CAT (μM H_2O_2/min/mg)	1409±67	1496±103	1518±69
GSH (mM/g)	24.7±1.7	32.7±2*	22.2±0.5
CD (nM/mg)	44.3±2.3	46.3±6	41.0±1.9
TBARS (nM/mg)	2.170±0.146	1.374±0.105**	1.707±0.113*
Kidney cortex			
SOD (U/mg)	0.085±0.004	0.072±0.008	0.081±0.004
GSH-Px (μMGSH/min/mg)	135±10	131±9	111±3
GR (μM NADPH/min/mg)	37.8±2.5	41.8±2.8	36.9±3.4
CAT (μM H_2O_2/min/mg)	556±42	769±43*	614±26
GSH (mM/g)	17.0±1.1	22.5±0.5*	15.7±0.6
CD (nM/mg)	23.8±1.3	27.8±3.1	24.3±2
TBARS (nM/mg)	1.345±0.075	1.267±0.099	1.291±0.056

* and ** denote P<0.05 and P<0.005, respectively, when compared to SHR control.

Phenotyping of congenic sublines

Table 1 shows biochemical and metabolic phenotypes in SHR versus SHR.BN-*Folr1* and SHR.BN-*Folh1* congenic sublines. As can be seen, both sublines had similar levels of plasma folate as the SHR. On the other hand, both sublines showed significantly reduced renal folate clearance that was associated with reduced cysteine and homocysteine levels as well as reduced levels of cysteinyl-glycine and γ-glutamylcysteine when compared to the SHR. In addition, both sublines had significantly reduced plasma glucose and insulin which suggested increased sensitivity of tissues to insulin action, however, there was no difference in basal and insulin stimulated glycogenesis when both lines were compared to the SHR. Furthermore, both sublines had significantly lower relative mass of epididymal fat which suggests reduced adiposity.

Parameters of oxidative stress

Table 2 shows that relative folate deficiency was associated with alterations in antioxidant enzyme activities especially in the liver and to the lesser extend in the kidney. The liver of both SHR.BN-*Folr1* and SHR.BN-*Folh1* sublines showed protection against oxidative tissue damage as reflected by reduced concentrations of lipoperoxidation products measured as conjugated dienes and TBARS.

Discussion

In the current study, we tested the separate effects of *Folh1* and *Folr1* gene variants on folate and sulfur amino acid metabolism and on parameters of glucose and lipid metabolism. We found that *Folh1* allele of the BN origin, contrary to the SHR allele, is weakly but consistently expressed in the small intestine. *Folh1* gene is located at the peak of QTL linkage for plasma cysteine levels and analysis of congenic sublines demonstrated that both *Folr1* and *Folh1* BN alleles were associated with reduced homocysteine and cysteine levels, and with amelioration of insulin resistance and decreased adiposity.

It has been reported that *Folh1* mRNA and protein are not expressed in the intestine and pancreas isolated either from Sprague-Dawley outbred stock or LEW inbred strain (Shafizadeh and Halsted 2007, Rovenská *et al*. 2008) and it was suggested that dietary polyglutamyl folates in rats are hydrolyzed by pancreatic γ-GH (γ-glutamyl hydrolase) and instead of *Folh1* (Shafizadeh and Halsted 2007). However, our results showed consistent intestinal mRNA expression of *Folh1* in SHR-1 congenic strain. As can be seen in Figure 1, the expression of *Folh1* in the SHR was negligible and it is possible that strains of Wistar origin, including LEW and

Sprague-Dawley as used in the former studies (Rovenská *et al*. 2008, Shafizadeh and Halsted 2007) and SHR used in the current study, do not express *Folh1* in the intestine in significant amount while unrelated BN strain shows weak but consistent expression. Thus it is possible that *Folh1* allele of the BN origin actually contributes to hydrolysis of polyglutamyl folates and absorption of folates from the intestine. On the other hand, Western blot analysis showed similar weak expression of FOLH1 protein in both SHR and SHR-1 strains.

Sequence analysis of the *Folh1* gene revealed 2 amino acid substitutions, the p.R15G and the p.T545N which is more conserved: human, mouse and BN rats have threonine while the SHR has asparagine and the mutation is within the catalytic domain of the gene. It is not clear whether these mutations affect downregulation of *Folh1* expression in the SHR.

Recently, we identified mutated *Folr1* as a quantitative trait gene using transgenic rescue experiment (Pravenec *et al.* 2016). However, this transgenic experiment does not provide evidence against a possible role of *Folh1* in folate metabolism and disturbances of sulfur amino acids and lipid and glucose parameters. It is possible that transgenesis of *Folr1* "rescued" both downregulated *Folr1* and *Folh1* SHR alleles. Phenotyping of SHR.BN-*Folr1* and SHR.BN-*Folh1* congenic sublines provided evidence that increased expression of both *Folr1* and *Folh1* alleles of the BN origin was associated with reduced cysteine and homocysteine levels, decreased adiposity and amelioration of insulin resistance. It can be concluded that the SHR is genetically predisposed to relative folate deficiency due to both reduced folate renal reabsorption and folate intestinal absorption, which is associated with hypercysteinemia, increased adiposity and insulin resistance.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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