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Genetically Determined Folate Deficiency Is Associated With Abnormal Hepatic Folate Profiles in the Spontaneously Hypertensive Rat

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Summary

Increased levels of plasma cysteine are associated with obesity and metabolic disturbances. Our recent genetic analyses in spontaneously hypertensive rats (SHR) revealed a mutated *Folr1* (folate receptor 1) as the quantitative trait gene associated with diminished renal *Folr1* expression, lower plasma folate levels, hypercysteinemia, hyperhomocysteinemia and metabolic disturbances. To further analyse the effects of the *Folr1* gene expression on folate metabolism, we used mass spectrometry to quantify folate profiles in the plasma and liver of an SHR-1 congenic strain, with wild type *Folr1* allele on the SHR genetic background, and compared them with the SHR strain. In the plasma, concentration of 5-methyltetrahydrofolate (5mTHF) was significantly higher in SHR-1 congenic rats compared to SHR (60±6 vs. 42±2 nmol/l, P<0.01) and 5mTHF monoglutamate was the predominant form in both strains (>99 % of total folate). In the liver, SHR-1 congenic rats showed a significantly increased level of 5mTHF and decreased concentrations of dihydrofolate (DHF), tetrahydrofolate (THF) and formyl-THF when compared to the SHR strain. We also analysed the extent of folate glutamylation in the liver. Compared with the SHR strain, congenic wild-type *Folr1* rats had significantly higher levels of 5mTHF monoglutamate. On the other hand, 5mTHF penta- and hexaglutamates were significantly higher in SHR when compared to SHR-1 rats. This inverse relationship of rat hepatic folate polyglutamate chain length and folate sufficiency was also true for other folate species. These results strongly indicate that the whole body homeostasis of folates is substantially impaired in SHR rats compared to the SHR-1 congenic strain and might be contributing to the associated metabolic disturbances observed in our previous studies.

Key words

Spontaneously hypertensive rat • Folate deficiency • Folate profiling • Folate glutamylation • Hepatic steatosis

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Introduction

The spontaneously hypertensive rat (SHR) is one of the most widely used animal models of increased blood pressure and associated metabolic disturbances. In our previous studies, we found that the SHR harbors a 5.7-kb deletion in the promoter region of the *Folr1* (folate receptor 1) gene on chromosome 1 that is associated with reduced renal expression of the gene, decreased renal folate reabsorption and lower circulating folate levels, hypercysteinemia, and hyperhomocysteinemia when compared to SHR-1 congenic rats with the wild-type *Folr1* gene (Pravenec *et al.* 2016). These

disturbances in folate homeostasis in SHR are associated with higher body weight because of increased adiposity and with increased ectopic fat accumulation in liver and muscle when compared to the SHR-1 congenic strain. The increased fat accumulation in the SHR was associated with reduced sensitivity of skeletal muscle to insulin action (Pravenec *et al.* 2016). The mechanisms connecting folate deficiency, hypercysteinemia and hyperhomocysteinemia with disturbances of lipid metabolism are not fully understood and may include an abnormal methylation status (Obeid *et al.* 2009, Carter *et al.* 2016). Folates are pteroyl(poly)glutamate derivatives with various one-carbon moieties at the pterine ring (Shane 2010). Humans are unable to synthesize folates and depend on their production by plants and to some extent possibly also by the gut microbiota. Dietary polyglutamylated folates are deglutamylated in the intestine, absorbed predominantly by the proton-coupled folate transporter. Four additional folate transporters with different expression pattern in various organs provide multiple routes for interorgan transport of folates predominately in the form of monoglutamylated 5-methyl-tetrahydrofolate (5mTHF). Both the retention of folates in the cells and their conversion to functional cofactors require polyglutamylation (Shane 2010, Lin *et al.* 1994). It should be noted that the folylpolyglutamates are much more effective substrates for one-carbon transfer reactions than the monoglutamylated folate species (Shane 2010). A number of enzymes participate in intracellular metabolism of one-carbon (C-1) moieties attached to the polyglutamylated tetrahydrofolate (THF) residue. These C-1 moieties are used to synthesize vital compounds: 5mTHF remethylates homocysteine back to methionine and subsequently to S-adenosylmethionine, 10-formylTHF and 5, 10-methyleneTHF are used for synthesis of purines and of thymidylate, respectively.

The mechanisms by which folate deficiency in SHR and in mice deficient in the methylenetetrahydrofolate reductase activity elicits disturbed lipid metabolism are only partially understood (Christensen *et al.* 2015, Pravenec *et al.* 2016). One mechanism may result from reduced availability of folate for homocysteine remethylation and deficiency of S-adenosylmethionine with accumulation of S-adenosylhomocysteine. Deficiency of S-adenosylmethionine and accumulation of S-adenosylhomocysteine could lead to reduced production of phosphatidylcholine which is an essential factor for very low-density lipoprotein assembly and transport of triglycerides out of the liver. The attendant disturbances in hepatic lipid transport could in turn be contributing to ectopic accumulation of fat in liver. In the current study, we measured the relative abundance of folate species in plasma and liver and the extent of folate glutamylation in the liver in SHR versus SHR-1 congenic rats to analyse how genetically determined folate deficiency affects folate metabolism and whether these disturbances may be one of the underlying mechanisms connecting low folate status with hepatic steatosis.

Materials and Methods

Animals

SHR/OlaIpcv rats (referred to as the SHR strain) and SHR.BN-*D1Rat272*/*Igf2* congenic rats (referred to as the SHR-1 congenic strain) (St. Lezin *et al.* 1997), males at the age of 3 months, N=6 per group, were housed in an air-conditioned animal facility and allowed free access to food (diet 1314, Altromin, Germany) and water. The SHR congenic strain was derived by a selective breeding protocol in which a segment of chromosome 1 from the normotensive BN/CrL strain was transferred onto the genetic background of the progenitor SHR. The coat color (*C*) and a microsatellite marker within the gene encoding insulin-like growth factor 2 (*Igf2*) were used for selection of heterozygous carriers in each back-cross generation. After 10 generations of selective back-crossing to the SHR progenitor strain, the differential chromosome segment was fixed using the markers *D1Mit3* (a microsatellite marker that maps close to *C*) and *Igf2* and maintained in the homozygous state by brother × sister mating and selective inbreeding of the offspring. 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.

Quantification of folate one-carbon metabolism (FOCM) intermediates by mass spectrometry

Distribution of differentially substituted folate species and the extent of glutamylation in plasma and liver of SHR (n=10) and SHR-1 (n=9) 3-month-old males were determined. Analysis of multiple folates was performed by UPLC-MS/MS as described previously (Pai

et al. 2015, Leung *et al.* 2017). Folate sample buffer containing 20 mM ammonia acetate, 0.1 % ascorbic acid, 0.1 % citric acid and 100 mM DTT at pH 7 was added to liver tissue or at equal volume to plasma. Liver samples were homogenised by sonication for 10 s using a hand-held sonicator at 60 % amplitude. Protein was removed by precipitation with addition of 2 sample volumes of acetonitrile and centrifugation for 15 min at 12,000 × g and 4 °C. Supernatants were transferred to fresh tubes, lyophilised and stored at -80 °C prior to analysis. Lyophilised plasma samples were resuspended in 30 µl water (milli-Q) and centrifuged for 5 min at 12,000 × g at 4 °C. Supernatants were transferred to glass sample vials for UPLC-MS/MS analysis. Metabolites were resolved by reversed-phase chromatography using an Acquity UPLC BEH C18 column (50 mm×2.1 mm; 1.7 µm bead size (Waters Corporation, UK). Solvents for UPLC were: Buffer A, 5 % methanol, 95 % Milli-Q water and 5 mM dimethylhexylamine at pH 8.0; Buffer B, 100 % methanol. The column was equilibrated with 95 % Buffer A: 5 % Buffer B. The sample injection volume was 20 µl. The UPLC protocol consisted of 95 % Buffer A: 5 % Buffer B for 1 min, followed by a gradient of 5-60 % Buffer B over 9 min and then 100 % Buffer B for 6 min before re-equilibration for 4 min. The metabolites were eluted at a flow rate of 200 nl/min. The UPLC was coupled to a XEVO-TQS mass spectrometer (Waters Corporation, UK) operating in negative-ion mode using the following settings: capillary 2.5 kV, source temperature 150 °C, desolvation temperature 600 °C, cone gas flow rate 150 l/h and desolvation gas flow rate 1,200 l/h. Folates were measured by multiple reaction monitoring (MRM) with optimised cone voltage and collision energy for precursor and product ions as described (Cabreiro *et al.* 2013, Leung *et al.* 2013). The plasma samples were spiked with a known concentration of methotrexate (MTX) as internal standard at 0.1 pmol/µl plasma to determine the folate concentration in plasma (pmol/µl). For the liver, the folate distribution is calculated as relative amount of total folate in the sample and is expressed as percentage of the total folate. Using buffer at pH 7, some abiotic conversion of CH_2-THF to THF occurs (maximal 20 % in a tissue matrix) but sensitivity is significantly greater than at pH 10 (which stabilises CH_2-THF) (Pai *et al.* 2015).

Statistical analysis

Summary results are expressed as mean ± SEM. Individual groups were compared by unpaired Student *t*-test. Normality of distribution was tested by Shapiro-Wilk method. Statistical significance was defined as $P<0.05$.

Results

Folate profiling

In the plasma, the concentration of 5-methyl-tetrahydrofolate (5mTHF) was significantly higher in SHR-1 congenic rats compared to SHR rats (60±6 vs. 42±2 nmol/l, $P<0.01$) and 5mTHF monoglutamate was the predominant form in both strains (>99 % of total folate). The folate profile in the liver also differed between strains (Fig. 1). The SHR-1 congenic rats had significantly increased proportion of 5mTHF and decreased percentages of DHF, THF, and formyl-THF when compared to SHR. 5mTHF was the predominant form in both strains. Most of the hepatic 5mTHF was in the form of monoglutamate (>99 %), and the relative proportion of monoglutamated 5mTHF was significantly higher in SHR-1 than SHR liver (Fig. 1). On the other hand, the relative proportion of 5mTHF penta- and hexaglutamates were significantly reduced in SHR-1 compared with SHR rat liver (Fig. 2). The same was true for other folate species: SHR-1 rats when compared to SHR exhibited significantly reduced percentages of pentaglutamyl and/or hexaglutamyl forms of DHF, THF, methenyl-THF, and methylene-THF in liver. These results demonstrate that there is an inverse relationship of rat liver folate polyglutamate chain length and folate sufficiency.

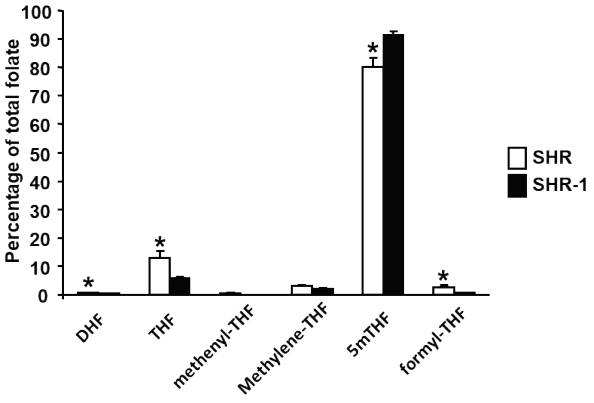

Fig. 1. Folate profiling in the liver shown as percentage of total folate. SHR-1 congenic rats (solid bars) had significantly increased proportion of 5mTHF and decreased percentage of DHF, THF, and formyl-THF when compared to SHR (open bars). 5mTHF was the predominant form in both strains. * denotes $P<0.05$.

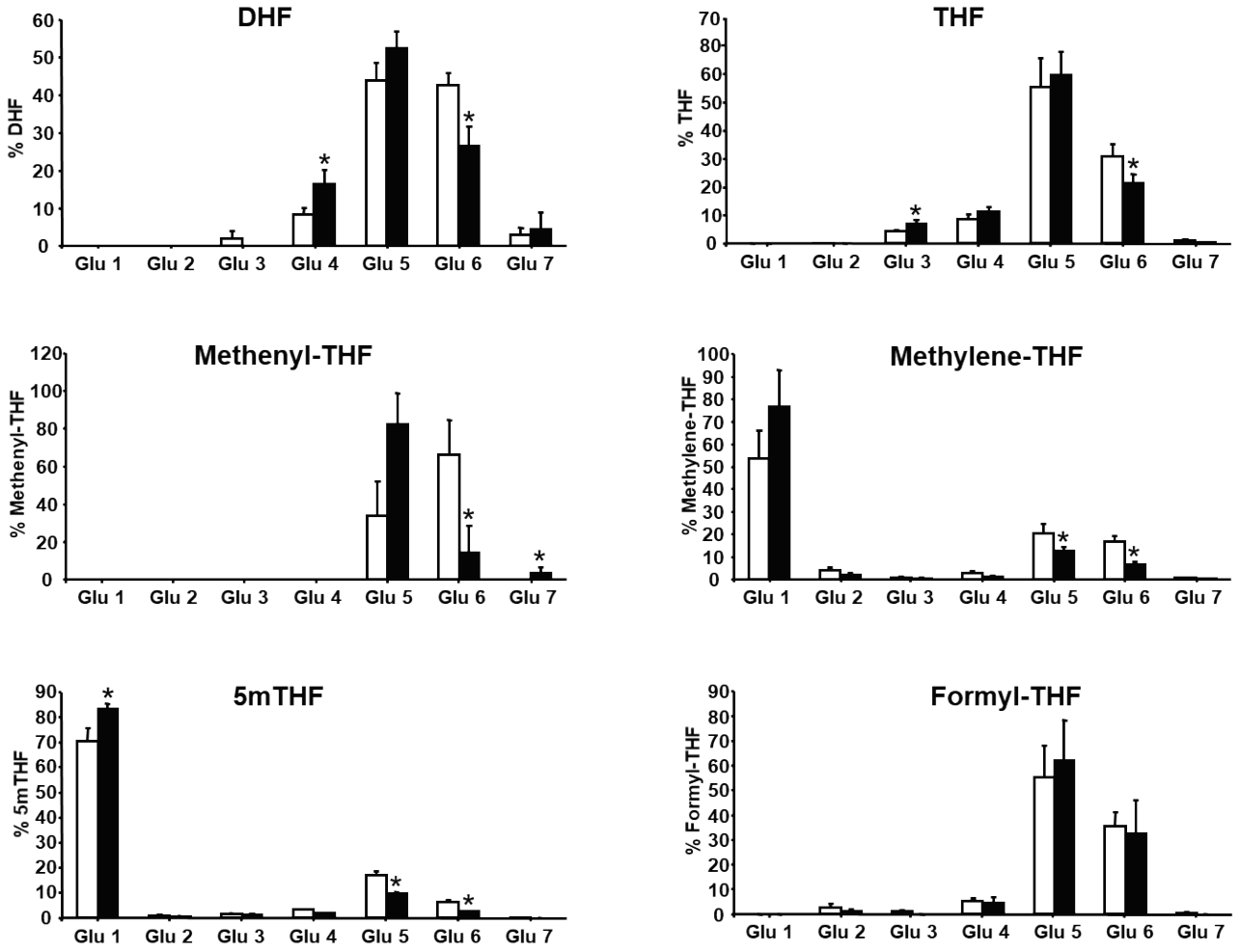

Fig. 2. Distribution of differentially substituted folate species and the extent of glutamylation in the liver from SHR (open bars) and SHR-1 congenic rats (solid bars) expressed as percentage of individual folate species concentrations. Most of the hepatic 5mTHF was in the form of monoglutamate and congenic rats had significantly higher percentage when compared to the SHR. On the other hand, 5mTHF penta- and/or hexaglutamates were significantly reduced in congenic rats versus the SHR. The same was true for other folate species. * denotes $P<0.05$.

Discussion

Folate metabolism plays a fundamental role in key cellular functions including nucleotide biosynthesis and methylation. Disturbances in folate homeostasis can be estimated from changes of downstream biomarkers such as homocysteine concentration, DNA methylation and uracil incorporation. On the other hand, folate profiling could provide direct information about the abundance of individual folate metabolites that may reflect disturbances of particular steps. In the current study, we analysed the effect of alteration in total abundance of folates on the folate profile by analysing plasma and liver of SHR rats that have a relative folate deficiency (owing to *Folr1* mutation) compared to the SHR-1 congenic strain, which carries a wild type *Folr1* allele (Pravenec *et al.* 2016). We observed significantly lower abundance of plasma and total hepatic 5mTHF in the SHR when compared to congenic SHR-1 rats. This is consistent with our previous findings that mutation of *Folr1* in SHR is associated with reduced renal expression of *Folr1* mRNA, decreased renal folate reabsorption and lower circulating folate levels. Interestingly, although total and monoglutamyl 5mTHF was lower in abundance in the liver of SHR rats, the 5mTHF penta- and hexaglutamates were significantly higher in the SHR compared with SHR-1 congenic rats. The same trend was observed for other folate species, including DHF, THF, methenyl-THF, and methylene-THF polyglutamates (Fig. 2). We hypothesise that this inverse relationship of rat liver folate polyglutamate chain length and folate sufficiency reflects the fact that the synthesis of long-chain folylpolyglutamates in the liver occurs at a slow rate and is limited by competition with the

entering pteroylmonoglutamate and shorter-chain folylpolyglutamates, which are preferred substrates for folylpolyglutatamate synthetase. It has been reported that under folate-restricted conditions, this competition is decreased (Cassady *et al.* 1980, Varela-Moreiras *et al.* 1992). Thus the increased relative abundance of folates with more than five glutamate residues in tissues from rats subject to folate deficiency is consistent with the possibility that these folates undergo slower turnover to allow the addition of extra glutamate residues. Accordingly, the increased relative abundance of DHF, THF and formyl-THF in the liver of SHR versus SHR-1 congenic rats is due to a higher percentage of penta- and hexaglutamate forms while the lower hepatic proportion of 5mTHF monoglutamate in SHR versus SHR-1 rats is likely due to lower cellular uptake, increased polyglutamylation and apparently reduced folate turnover. Lower abundance of 5mTHF has been found to result in decreased contribution of methyl groups to the methylation cycle and predisposition to hepatic steatosis (Obeid *et al.* 2009).

In conclusion, our results strongly indicate that the whole body homeostasis of folates is substantially impaired in the SHR compared with the SHR-1 congenic strain. This may be in part responsible for the associated metabolic disturbances, including increased serum levels of cysteine and homocysteine, increased adiposity, ectopic fat accumulation in liver and muscle, reduced muscle insulin sensitivity, and increased blood pressure that are observed in the SHR strain (Pravenec *et al.* 2016).

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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