Mutant *Wars2* Gene in Spontaneously Hypertensive Rats Impairs Brown Adipose Tissue Function and Predisposes to Visceral Obesity

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Received November 17, 2017
Accepted November 30, 2017

Summary

Brown adipose tissue (BAT) plays an important role in lipid and glucose metabolism in rodents and possibly also in humans. Identification of genes responsible for BAT function would shed light on underlying pathophysiological mechanisms of metabolic disturbances. Recent linkage analysis in the BXH/HXB recombinant inbred (RI) strains, derived from Brown Norway (BN) and spontaneously hypertensive rats (SHR), identified two closely linked quantitative trait loci (QTL) associated with glucose oxidation and glucose incorporation into BAT lipids in the vicinity of *Wars2* (tryptophanyl tRNA synthetase 2 (mitochondrial)) gene on chromosome 2. The SHR harbors L53F WARS2 protein variant that was associated with reduced angiogenesis and *Wars2* thus represents a prominent positional candidate gene. In the current study, we validated this candidate as a quantitative trait gene (QTG) using transgenic rescue experiment. SHR-*Wars2* transgenic rats with wild type *Wars2* gene when compared to SHR, showed more efficient mitochondrial proteosynthesis and increased mitochondrial respiration, which was associated with increased glucose oxidation and incorporation into BAT lipids, and with reduced weight of visceral fat. Correlation analyses in RI strains showed that increased activity of BAT was associated with amelioration of insulin resistance in muscle and white adipose tissue. In summary, these results demonstrate important role of *Wars2* gene in regulating BAT function and consequently lipid and glucose metabolism.

Key words

Brown adipose tissue • Spontaneously hypertensive rat • Quantitative trait loci • Transgenic • *Wars2* gene • Mitochondrial proteosynthesis

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Introduction

Metabolic syndrome is characterized by clustering of several risk factors including insulin resistance in skeletal muscle and adipose tissue, dyslipidemia and hypertension. Growing evidence indicates that brown adipose tissue (BAT) plays an important role in the pathogenesis of metabolic disturbances both in rodent models and in humans (Bartelt *et al.* 2011, Vijgen *et al.* 2011, Virtanen 2016). Identification of genes that regulate BAT function would shed light on underlying pathophysiological mechanisms of the metabolic syndrome. Recently, we performed linkage analyses of physiological phenotypes in BAT in BXH/HXB recombinant inbred (RI) strains derived from SHR (spontaneously hypertensive rat) and BN (Brown Norway) progenitors. These linkage studies identified two closely linked quantitative trait loci (QTL) on chromosome 2 associated with glucose oxidation (the peak of QTL linkage at position 181 Mb) and glucose incorporation into BAT lipids (the peak of QTL linkage at position 200 Mb) in the vicinity of *Wars2* (tryptophanyl tRNA synthetase 2 (mitochondrial)) gene (at position 201 Mb) (Pravenec *et al.* 2017). The SHR harbors mutant *Wars2* allele, which codes for an L53F

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WARS2 protein variant within the ATP-binding motif and has been identified as a genetic determinant that predisposes the SHR to reduced angiogenesis in the heart and possibly in other tissues (Wang *et al.* 2016). The *Wars2* thus represents a prominent positional candidate gene for transgenic rescue experiments to identify QTL associated with BAT function at the molecular level. In the current study, we tried to identify the *Wars2* as a quantitative trait gene and to search for pathophysiological mechanisms underlying the associated phenotypes. Correlation analysis in RI strains was used to evaluate the association of BAT activity with insulin resistance in skeletal muscle and white adipose tissue.

Materials and Methods

Animals

The SHR/OlaIpcv strain (referred to as the SHR), SHR.BN-*D2Rat171*/*D2Arb24* congenic strain with wild type *Wars2* (referred to as SHR-2) (Pravenec *et al.* 2001) and the new SHR transgenic strain expressing wild type *Wars2* gene were housed in a facility with constant temperature 23 °C and 12 h light/dark cycle and allowed free access to standard laboratory chow and water. Transgenic SHR/Ola-Tg(*EF1a-Wars2*) (referred to as the transgenic SHR-*Wars2*) strain was derived by microinjecting fertilized eggs with a mix of the Sleeping Beauty construct containing BN *Wars2* cDNA under control of the universal EF-1α promoter and mRNA of the SB100X transposase (Ivics *et al.* 2014). Transgenic rats were detected using PCR with the following primers: *Wars2*-F 5'-TGT GCT ACA AGT CCA CAC AC-3' and *Wars2*-R 5'-GCA GAA GGG TCA CGA AGA GA-3'. Biochemical and metabolic phenotypes in both strains were assessed in 3-month-old non-fasted male rats (n=8 per strain). 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.

Glucose utilization in BAT for oxidation and incorporation into lipids

Following decapitation in the non-fasted state, interscapular BAT was dissected and incubated for 2 h in Krebs-Ringer bicarbonate buffer with 5 mmol/l glucose, 0.1 μCi [U-^{14}C] glucose/ml and 2 % bovine serum albumin, gaseous phase 95 % O_2 and 5 % CO_2. Glucose oxidation was determined in BAT by measuring the incorporation of [U-^{14}C] glucose into CO_2. For measurement of incorporation of radiolabeled glucose into lipids, at the end of incubation, BAT was removed from media, rinsed in saline, and transferred into chloroform:methanol (2:1) where lipids were extracted and radioactivity measured.

Gene expression determined by real-time PCR

Total RNA was extracted from the BAT using Trizol reagent (Invitrogen, Waltham, USA) and cDNA was prepared and analyzed by real-time PCR testing using QuantiTect SYBR Green reagents (Qiagen, Inc., Venlo, Netherlands) on an Opticon continuous fluorescence detector (MJ Research, Waltham, USA). Gene expression levels were normalized relative to the expression of the peptidylprolyl isomerase A (*Ppia*) (cyclophilin) gene, which served as the internal control. The results were determined in triplicates. The following primers were used: *Wars2*-F 5'-GTG CTA CAA GTC CAC ACA CG-3'; *Wars2*-R 5'-GCA GAA GGG TCA CGA AGA GA-3'; *Ppia*-F 5'-AGC ATA CAG GTC CTG GCA T-3'; *Ppia*-R 5'-TCA CCT TCC CAA AGA CCA C-3'.

Tissue homogenates, isolated mitochondria and culture of rat skin fibroblasts

Preparation of tissue homogenates and isolation of heart mitochondria were carried as previously described (Pecinová *et al*. 2011), proteins were determined by the Bradford method (1976), using BSA as standard. Primary rat skin fibroblasts were prepared from 3 weeks old SHR and SHR-2 congenic animals according to Seluanov *et al.* (2010). Established cultures of skin fibroblasts were then maintained and subcultured at 37 °C and 5 % CO_2 in air in DMEM medium (Life Technologies, Waltham, USA) that was supplemented with 10 % fetal calf serum (Sigma, St. Louis, USA) and penicillin/streptomycin solution (Life technologies).

Western blotting

Samples of fibroblasts or tissue homogenates were denatured at 56 °C for 15 min in a sample lysis buffer (2 % (v/v) 2-mercaptoethanol, 4 % (w/v) SDS, 50 mM Tris-HCl, pH 7.0, 10 % (v/v) glycerol, 0.017 % (w/v) Coomassie Brilliant Blue R-250) and Tricine SDS-PAGE was performed on 10 % (w/v) polyacrylamide slab gels. The gels were blotted onto a PVDF membrane (Immobilon-P, Merck Millipore) by semidry electrotransfer at 0.8 mA/cm^2 for 1 h. Membranes were

blocked in 5 % non-fat dried milk dissolved in Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 1 h at room temperature. Specific primary antibodies were used to assess the content of respiratory chain enzymes (SDHA, a subunit of complex II – ab14715; COX1, an mtDNA-encoded subunit of complex IV – ab14705; F_1-α, a subunit of complex V – ab110273, all from Abcam, Cambridge, UK), mitochondrial content (porin – a kind gift from Professor de Pinto) and WARS2 protein (SC-22852, from Santa Cruz Biotechnology, Dallas, USA). For quantitative detection, the corresponding infra-red fluorescent secondary antibodies (Alexa Fluor 680, Life Technologies; IRDye 800, Rockland Immunochemicals, Limerick, USA) diluted in TBS supplemented with 0.1 % (v/v) Tween-20 were used. The fluorescence was detected using ODYSSEY infra-red imaging system (LI-COR Biosciences, Lincoln, USA) and the signal was quantified using Aida 3.21 Image Analyzer software.

Metabolic pulse-chase labelling of mtDNA encoded proteins in SHR-2 congenic rats

The incorporation of ^{35}S-Met and ^{35}S-Cys into mtDNA-encoded proteins was investigated in the presence of emetine, an inhibitor of translation of nuclear-encoded proteins on cytosolic ribosomes. Cells were washed three times with PBS. 15-min incubation in DMEM medium without Met and Cys was followed by addition of emetine (100 μg/ml). After 15 min, the medium was exchanged for DMEM medium supplemented with emetine and ^{35}S-Protein Labelling Mix (^{35}S-Met+Cys, Perkin Elmer NEG072; 200 μCi/ml). Cells were incubated for 3 h at 37 °C, then 250 μM cold Met and Cys was added. After 15 min at 37 °C, cells were washed twice with PBS + 250 μM cold Met and Cys and harvested by trypsin digestion (washed twice with PBS supplemented with a protease inhibitor cocktail – Sigma P8340). Samples for SDS-PAGE were prepared as described above, resolved on 10 % gels and transferred to a PVDF membrane. The signal of incorporated radioactive Met and Cys was detected using Pharos FX^{TM} Plus Molecular Imager (Bio-Rad Laboratories, Hercules, USA) and quantified using Aida 3.21 Image Analyzer software. Afterwards, the membrane was probed with specific antibodies (see Western blotting for details) and radioactive signals of mitochondrial proteins were normalized to the protein content of porin, a mitochondrial marker, as determined on Western blots.

Respiration measurements

Oxygen consumption was measured at 30 °C as described before (Pecina *et al*. 2003) using Oxygraph-2k (Oroboros, Innsbruck, Austria). The isolated mitochondria (0.05-0.1 mg/ml) were suspended in 2 ml of KCl medium (80 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM K-Pi, 0.5 mg/ml BSA, pH 7.4). For measurements, 10 mM glutamate, 2.5 mM malate, 10 mM succinate and 1.5 mM ADP were used. The oxygen consumption was expressed in pmol oxygen/s/mg protein, respiratory control index (RCI) was calculated as a ratio between ADP-stimulated respiration and respiration without ADP for the substrate used.

Measurement of capillary number

Tissue samples of BAT were fixed in 10 % neutral buffered formalin (Sigma-Aldrich). 5 μm thick sections were stained by isolectin IB4 (Isolectin GS-IB4 Alexa Fluor 568 conjugate, ThermoFisher Scientific) and by anti-perilipin A antibody (Abcam) followed by incubation with Alexa Fluor 633 anti-goat IgG secondary antibody (ThermoFisher Scientific) and DAPI, or by anti-sodium potassium ATPase antibody (Abcam) followed by incubation with ABC kit (Vector Laboratories, Burlingame, USA) and DAB (Sigma-Aldrich). For each treatment, a positive control with a known positivity for specific antibody and a negative control with omitted primary antibody were included. Digital images were captured using an Olympus AX70 light microscope and a DP 70 camera (Olympus, Japan) or Leica SP8 AOBS WLL MP confocal microscope. Vascularization was assessed by semi-automatic detection of isolectin-positive capillaries in 10 high-power fields related to number of adipocytes detected by autofluorescence.

Statistical analysis

The data are expressed as means ± SEM. Individual groups were compared by Student t-test. Normality of distribution was tested by Shapiro-Wilk method. Statistical significance was defined as $P<0.05$. Statistical analysis of gene expression data was performed using the REST XL program, which tests for significance using a randomization procedure (Pfaffl *et al*. 2002). Correlation analysis of glucose oxidation and incorporation into BAT lipids (Pravenec *et al*. 2017) with parameters of glucose and lipid metabolism in the BXH/HXB RI strains were performed using GeneNetwork database and online software (www.genenetwork.org) (Mulligan *et al*. 2017).

Results

In vivo functional studies

To identify the two closely linked QTL on chromosome 2 at the molecular level as the *Wars2* gene variant, we measured glucose oxidation and incorporation into BAT lipids in new SHR-*Wars2* transgenic rats. SHR-*Wars2* transgenic rats exhibited significantly higher expression of the wild type mRNA and WARS2 protein (Figs 1A and 1B) and increased glucose oxidation and incorporation into BAT lipids when compared to SHR rats (Fig. 1C). Increased glucose oxidation and incorporation into BAT lipids in SHR-*Wars2* transgenic rats versus SHR was associated with lower body weight (243±3 versus 282±2 g, P=0.000006) to which contributes reduced adiposity as suggested by decreased relative weight of epididymal fat in transgenic rats (0.630±0.024 versus 0.813±0.028 g/100 g body weight, P=0.0006). Together, these findings provide the evidence that the mutant *Wars2* is a quantitative trait gene (QTG) responsible for reduced glucose oxidation and glucose incorporation into BAT lipids in the SHR.

Effects of the mutant Wars2 gene on synthesis of mtDNA encoded proteins, respiration and enzyme activities

Mitochondrial proteosynthesis was measured in primary fibroblast culture from SHR strain and in SHR-2 congenic strain which harbors wild type *Wars2* allele (Fig. 2A). There was a general increase in mitochondrial proteosynthesis in SHR-2 congenic strain, as would be expected for moderate aminoacyl-tRNA synthetase defect in SHR that affects mitochondrial proteosynthesis in general. It reached statistical significance for several mtDNA-encoded subunits of respiratory chain complexes (Nd4, Cyt b, Nd1 and Nd2). Presumably, this reflects number of tryptophan residues in individual protein subunits, as significant differences were observed in proteins with the highest number of tryptophans (13 times in Nd4, 10 times in Cyt b). An analogous downregulation of mitochondrial protein synthesis was observed in SHR cardiomyocytes (data not shown). In addition, lower rate of mitochondrial protein synthesis apparently affected respiratory chain function as isolated mitochondria from SHR versus SHR-2 congenic rats showed significantly reduced ADP-stimulated respiration with NADH-dependent substrates (glutamate and malate), as well as the respective respiratory control index (Fig. 2B).

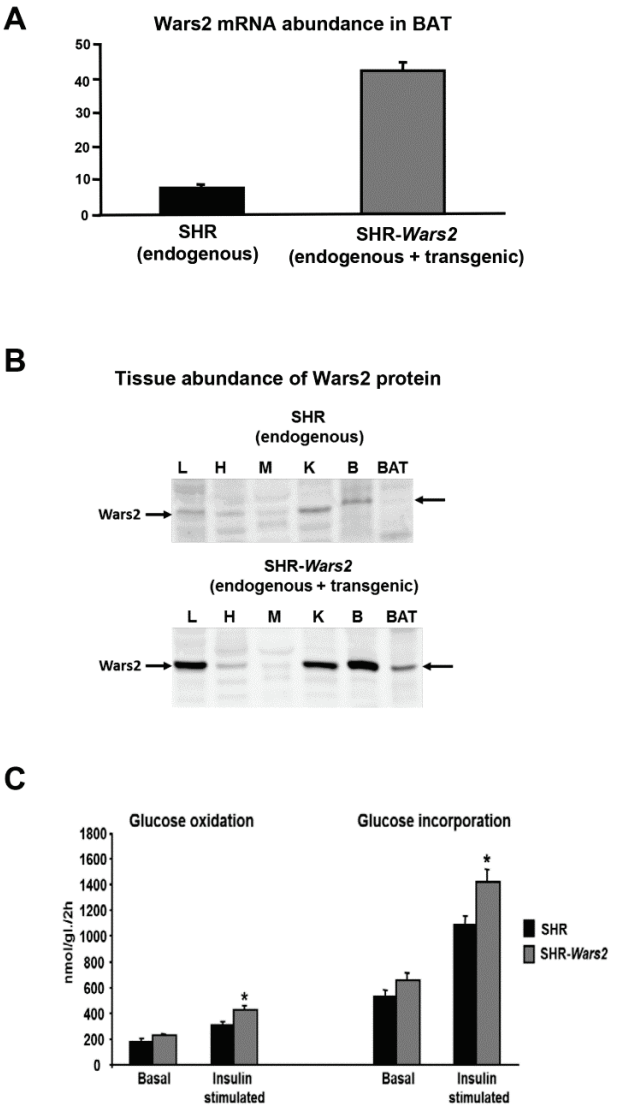

Fig. 1. Expression of *Wars2* in BAT isolated from SHR-*Wars2* transgenic rats and SHR rats and glucose oxidation and incorporation into BAT lipids. (**A**) *Wars2* mRNA abundance (n=4 per strain). (**B**) Western blot detected WARS2 protein tissue abundance. The methods detect both SHR-specific mutant type and wild type forms of *Wars2* (n=6 per strain). (**C**) SHR-*Wars2* transgenic rats with wild type *Wars2* exhibited increased glucose oxidation and incorporation into BAT lipids when compared to SHR controls (n=8 per strain). L – liver, H – heart, M – muscle, K – kidney, B – brain, BAT – brown adipose tissue.

Effects of Wars2 gene variants on capillary density in BAT

Since mutant *Wars2* was originally identified as a genetic determinant of reduced cardiac angiogenesis in the SHR (Wang *et al*. 2016), we tested whether decreased glucose oxidation and glucose incorporation into BAT lipids is due to reduced angiogenesis. No significant differences in capillary density were observed in BAT isolated from SHR and SHR-2 congenic rats (Fig. 3). These results suggest that mutant *Wars2* does not affect BAT glucose metabolism through reduced angiogenesis and decreased capillary density.

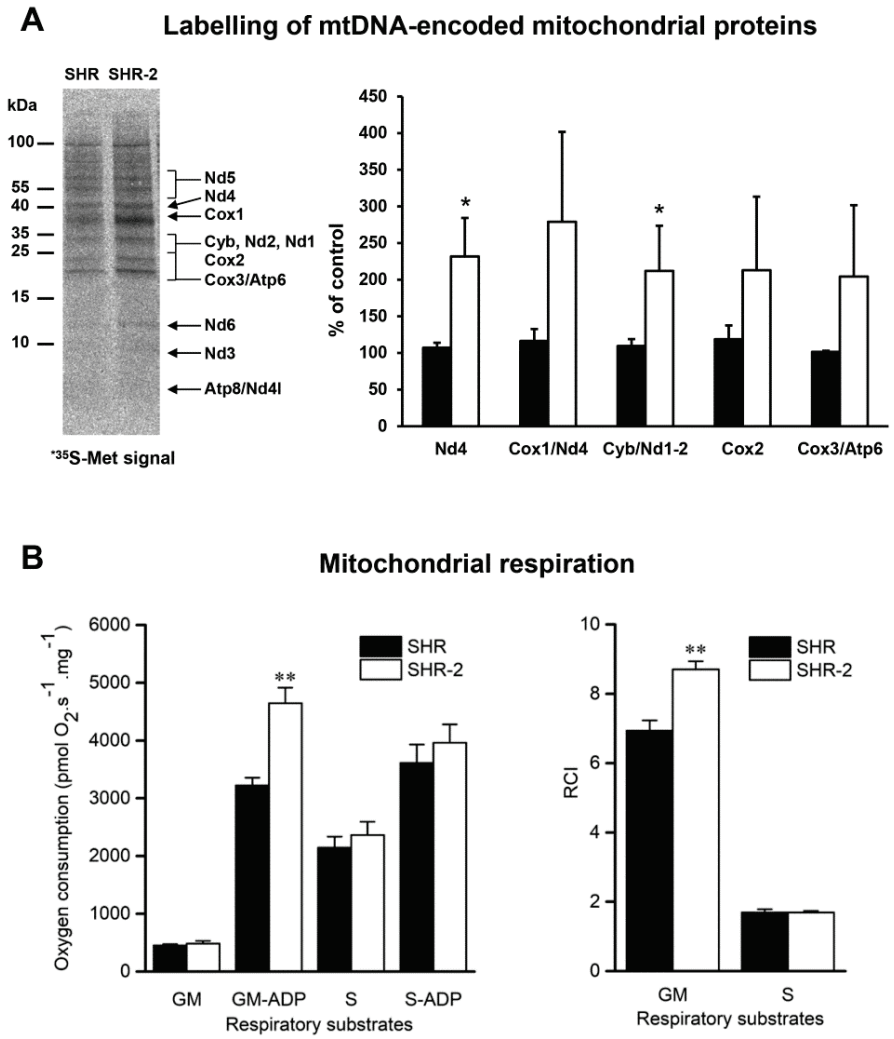

Fig. 2. Effects of *Wars2* gene variant on mitochondrial proteosynthesis and respiration. (**A**) Synthesis of mtDNA-encoded proteins in fibroblasts isolated from SHR with mutant *Wars2* gene and SHR-2 congenic rats with wild type *Wars2* gene. ^{*35}S methionine incorporation in the presence of emetine, an inhibitor of translation of nuclear DNA encoded proteins on cytosolic ribosomes. Specific labelling of mtDNA-encoded proteins (ND1, ND2, ND4 – subunits of respiratory chain complex I; COX2, COX3 – subunits of complex IV, ATP6 – subunit of ATP synthase) was normalized to mitochondrial porin content and expressed in % of SHR mean value (n=5 per strain). (**B**) Mitochondrial respiration using glutamate + malate (GM) or succinate (S) as a substrate was performed in the presence and absence of ADP and is expressed per mg protein. Respiratory control index (RCI) for the substrate used was calculated as a ratio between respiration in the presence and absence of ADP (n=5 per strain).

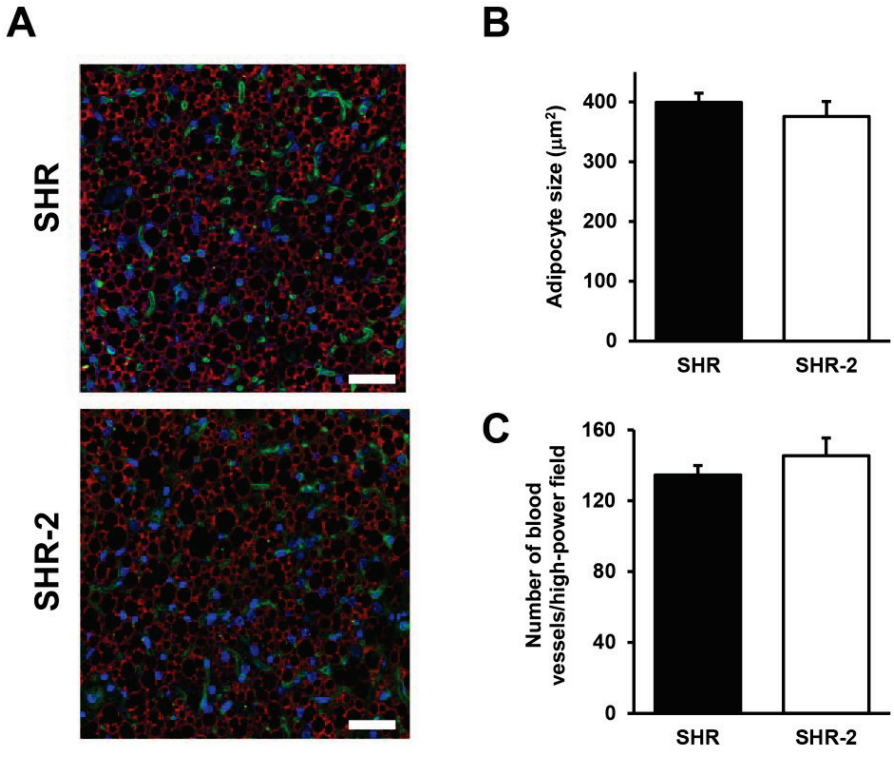

Fig. 3. Immunohistochemical characterization of brown adipose tissue of SHR and SHR-2 congenic rats. (**A**) Immunofluorescence analysis using isolectin IB4 (green; Alexa Fluor 568-conjugate) and antibody to perilipin A (red; Alexa Fluor 633 anti-goat IgG); nuclei counterstained by DAPI (blue). Scale bar, 20 μm. (**B**) The size of brown adipocytes was assessed using antibody to sodium-potassium ATPase; 800 objects per sample were analyzed (n=6). (**C**) Vascularization was assessed as isolectin-detected capillaries per high-power field; 6 microphotographs per sample were analyzed (n=6).

Table 1. Correlations of BAT traits with metabolic parameters in 30 RI strains.

Phenotype	r value	P value
Glucose oxidation in BAT		
Body weight	-0.60	0.0005
Adrenaline-stimulated lipolysis in epididymal fat (glycerol)	0.57	0.008
Adrenaline-stimulated lipolysis in epididymal fat (NEFA)	0.47	0.02
Insulin-stimulated glucose uptake in isolated WAT adipocytes	-0.40	0.04
Glucose incorporation into BAT lipids		
Insulin-stimulated glycogenesis in diaphragm	0.44	0.02
Basal glycogenesis in diaphragm	0.39	0.04

Correlation analyses of BAT phenotypes with metabolic traits in BXH/HXB recombinant inbred strains

As can be seen in Table 1, glucose oxidation in BAT correlated negatively with body weight and insulin stimulated glucose uptake in isolated WAT adipocytes but positively with stimulated lipolysis in WAT. Glucose incorporation into BAT lipids correlated positively with glucose incorporation into skeletal muscle glycogen (glycogenesis). Both increased lipolysis in white adipose tissue and enhanced glucose incorporation in skeletal muscle indicate the amelioration of insulin resistance.

Discussion

In the current studies, we performed *in vivo* transgenic rescue experiments to identify two closely linked QTL on chromosome 2 associated with glucose oxidation and glucose incorporation into BAT lipids as a variant of the *Wars2* gene. The SHR-*Wars2* transgenic rats exhibited significantly increased glucose oxidation and glucose incorporation into BAT lipids when compared to nontransgenic SHR. These results are consistent with differences in glucose oxidation and incorporation into BAT lipids observed previously in SHR-2 congenic rats with wild type *Wars2* allele versus SHR (Pravenec *et al*. 2017). Originally, the SHR *Wars2* mutant allele was identified as a genetic determinant of reduced cardiac angiogenesis when it was demonstrated that SHR-2 congenic rats had significantly higher capillary number when compared to SHR rats (Wang *et al*. 2016). Contrary to the heart tissue, the SHR *Wars2* mutant variant had no significant effect on BAT capillary number in the SHR-2 congenic versus SHR rats but was associated with a general reduction in the synthesis of mitochondrial proteins. Thus it is possible that reduced proteosynthesis in BAT mitochondria and associated reduced mitochondrial function (decreased respiration) is responsible for lower glucose oxidation and incorporation into BAT lipids.

Transgenic rescue experiments showed that wild type *Wars2* allele was associated with lower body weight to which contributed significantly reduced weight of visceral fat. Congruently, meta-analysis of 32 genome-wide association studies (GWAS) in humans revealed a significant association of single nucleotide polymorphisms (SNPs) within the TBX15-WARS2 haplotype with waist-to-hip ratio (Heid *et al*. 2010, Yoneyama *et al*. 2017). These associations were reproduced by Liu *et al.* (2014) and TBX15-WARS2 as a genetic determinant of regional fat distribution was mapped within a narrow region of ±250 kb (Liu *et al*. 2014). Unfortunately, no definitive functional SNPs were identified within this locus (Yoneyama *et al*. 2017). Although reduced visceral fat in SHR-*Wars2* rats is causally linked to the transgene expression, it is conceivable that in humans, the TBX15 gene variant might be responsible for the observed association with fat distribution. For instance, it has been demonstrated that TBX15 is differentially expressed in subcutaneous and visceral fat depos in humans and mice (Gesta *et al*. 2011); but this is also true for the WARS2 gene (Heid *et al*. 2010). Altogether these findings strongly suggest that TBX15-WARS2 haplotype in humans and *Wars2* gene in the SHR affect the amount of visceral fat.

Glucose oxidation in BAT associated with the *Wars2* gene correlated inversely with glucose incorporation into WAT adipocytes in RI strains (www.genenetwork.org). This result suggests that glucose was preferentially used for oxidation in BAT and not for lipogenesis and fat accumulation. Inverse correlation of glucose oxidation with body weight might reflect reduced adiposity due to a more effective

mitochondrial function in BAT. Glucose incorporation into BAT lipids in RI strains (www.genenetwork.org) was correlated with increased lipolysis of WAT and higher sensitivity of skeletal muscles to insulin action, i.e. these BAT traits were associated with amelioration of insulin resistance. These results suggest that *Wars2* gene, by affecting BAT function, plays an important role in glucose and lipid metabolism in the SHR model. Interestingly, expression of the *Wars2* gene in white adipose tissue in the mouse BXD recombinant inbred strains (derived from C57BL/6 and DBA/2 progenitors) is inversely correlated with obesity trait (body weight gain 17 to 18 days after feeding a high-fat diet, r=-0.87, P=0.00006) which is in agreement with our results that reduced *Wars2* expression might predispose to obesity (Brockmann *et al.*, unpublished results, www.genenetwork.org). Thus variants in *Wars2* gene are linked to obesity traits both in rodent models and in humans.

In summary, results of the current study provide the evidence for important role of *Wars2* gene in regulating BAT function and consequentially lipid and glucose metabolism in the SHR when SHR mutant variant is associated with reduced mtDNA proteosynthesis, lower mitochondrial function and predisposition to increased visceral fat accumulation.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the Czech Science Foundation (grant number 13-04420S) to MP.

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