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Nicotinamide nucleotide transhydrogenase (NNT) mRNA expression is related to human obesity

Running head: NNT and human obesity

John T. Heiker[1], Matthias Kern[1], Joanna Kosacka[1], Gesine Flehmig[2], Michael Stumvoll[1], Edward Shang[3], Tobias Lohmann[4], Miriam Dreßler[4], Peter Kovacs[5], Matthias Blüher[1,2], Nora Klöting[1,6]

[1]*Department of Medicine, Endocrinology and Diabetes, Universität Leipzig, Germany*
[2]*IFB AdiposityDiseases, Universität Leipzig, Leipzig, Germany*
[3] *Department of Surgery, University of Leipzig, Leipzig, Germany*
[4] *Municipal Clinic Dresden-Neustadt, Dresden, Germany*
[5]*Faculty of Medicine, Universität Leipzig, Leipzig, Germany*
[6]*IFB AdiposityDiseases, Junior Research Group 2 "Animal models of obesity", Universität Leipzig, Germany*

Corresponding author:
Nora Klöting
University of Leipzig
IFB AdiposityDiseases
Liebigstr. 21, D-04103 Leipzig, Germany
e-mail: nora.kloeting@medizin.uni-leipzig.de
Tel. (+49) 341-97-13401 Fax (+49) 341-9713409

Abstract

It has been proposed that a spontaneous deletion in the nicotinamide nucleotide transhydrogenase (*Nnt*) gene eliminating exons 7-11 in C57BL/6J (B6J) mice is associated with reduced glucose-stimulated insulin secretion *in vitro,* impaired glucose tolerance, higher epigonadal fat mass and altered susceptibility to diet induced obesity (DIO) of male B6J mice. A potential implication for NNT in human adipose tissue distribution has not been investigated so far. We therefore analyzed *NNT* mRNA expression in paired human samples of visceral (vis) and subcutaneous (sc) adipose tissue from 221 subjects with a wide range of BMI, insulin sensitivity and glucose tolerance.

NNT mRNA expression is significantly higher in visceral fat of obese patients and correlates with body weight, BMI, % body fat, visceral and sc fat area, waist and hip circumference as well as fasting plasma insulin. Multivariate linear regression analysis revealed visceral *NNT* expression as age and gender independent predictor of BMI, waist circumference, visceral fat area, and % body fat, but not fasting plasma insulin and 2h OGTT glucose. In conclusion, our data suggest a functional relevance of NNT in the development of human obesity and visceral fat distribution.

Introduction

Phenotypic differences among C57BL/6 mouse substrains have been reported with respect to behavior (1-3), glucose and insulin tolerance (4) as well as responsiveness to alcohol (5) and drugs (6-7). Several single nucleotide polymorphisms (SNP) differences were found between C57BL/6J (B6J) and C57BL/6N (B6N) strain (8) but the most prominent and investigated genetic variation between the core inbred mouse strains *B6J* and B6N relates to the nicotinamide nucleotide transhydrogenase (Nnt) gene on chromosome 13. Eukaryotic NNT functions as a homodimeric redox-driven proton pump catalyzing the reversible reduction of $NADP^+$ to NADPH and subsequent conversion of NADH into NAD^+ (9-10). Each subunit is composed of three principal domains: the first and third domains lie within the mitochondrial matrix and contain the NAD(H)- and NADP(H)-binding domains, respectively. The second consists of 14 transmembrane-spanning helices and harbors the proton-conducting pore (9). In the B6J strain, a missense (methionine to threonine) mutation, in combination with an in-frame 5 exon deletion mutation (eliminating four putative transmembrane helices) results in a truncated Nnt variant and markedly lower Nnt protein expression in liver and islets (11-13). Nnt activity has been linked to impaired glucose metabolism and insulin secretion. Besides a role in insulin secretion, the Nnt mutation has been investigated in context of diet induced obesity (DIO). Studies on high fat DIO have revealed differences between core substrains of C57BL/6, wild type Nnt expressing C57BL/6NTac and Nnt-mutant C57BL/6J regarding their ability to respond to HFD and develop DIO reflected by body weight gain and whole body fat mass (7). Recently, we reported phenotypical differences under high fat diet (HFD) conditions between C57BL/6NTac and C57BL/6JRj substrains and confirmed additional genetic disparity in terms of 11 SNP loci (8). The C57BL/6JRj strain was found to be protected against DIO independent from physical activity and food intake (8). Based on these data from *in vitro* and animal studies, we hypothesize that human adipose tissue NNT expression is related to parameters of obesity, fat distribution, insulin sensitivity and glucose tolerance. We

investigated whether *NNT* mRNA expression in human adipose tissue is primarily related to parameters of obesity and fat distribution or to metabolic traits including insulin sensitivity and glucose tolerance.

Methods and Procedures

Subjects

Paired samples of visceral and subcutaneous adipose tissue were obtained from 221 Caucasian men (N = 80) and women (N = 141) who underwent open abdominal surgery for cholecystectomy, appendectomy, weight reduction surgery, abdominal injuries or explorative laparotomy (Table 1). The age ranged from 18 to 89 years and body mass index from 13.8 to 71.0 kg/m^2. Sixty nine subjects had type 2 diabetes. All subjects had a stable weight with no fluctuations of more than 3% of the body weight for at least three months before surgery. Patients with severe conditions including generalized inflammation or end stage malignant diseases were excluded from the study. Samples of visceral and subcutaneous adipose tissue were immediately frozen in liquid nitrogen after explantation. The study was approved by the Ethics Committee of the University of Leipzig (Germany). All subjects gave written informed consent before taking part in the study.

Measures of body fat content

BMI was calculated as weight (in kg) divided by the square of height (in m). Waist and hip circumferences were measured, and the WHR was calculated. Percentage body fat was measured by dual-energy X-ray absorptiometry (DEXA). In addition, abdominal visceral and subcutaneous fat areas were calculated using computed tomography (CT) scans at the level of L4–L5 (14).

Analysis of human *NNT* gene expression

Human *NNT* mRNA was measured by quantitative real-time RT-PCR using the TaqMan assay (Hs00966097_m1) and hypoxanthine guanine phosphoribosyltransferase (HPRT,

Hs01003267_m1) as house-keeping gene and fluorescence was detected on an ABI PRISM 7000 sequence detector (Applied Biosystems, Darmstadt, Germany). Human *NNT* gene expression was determined by the standard curve method and normalized to the house-keeping gene HPRT as previously described (15-16). 1 µg of total RNA (TRIzol Reagent by Life Technologies, Grand Island, NY) from paired subcutaneous and visceral adipose tissue samples was reverse transcribed with standard reagents (Life Technologies, Grand Island, NY) as shown elsewhere (15-16). Quantitative real-time reverse transcription-PCR was performed for each sample in duplicate with total RNA, 1×TaqMan Universal Master Mix no AmpErase UNG, 6.25 units of murine leukemia virus reverse transcriptase (both from Applied Biosystems) and gene-specific primers-probe sets, using an ABI PRISM 7000 sequence detector (Applied Biosystems). cDNA samples were incubated in the ABI PRISM 7000 sequence detector for an initial denaturation at 95°C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95°C for 15 s, 60°C for 1 min, and 72°C for 1 min. Accuracy of RNA quantitation was optimized by gene-specific primer-probe sets that span intron-exon boundaries. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Data analysis and statistics

Data are given as means ± SD unless stated otherwise. Data sets were analyzed using a two-tailed paired *t* test or Mann-Whitney test, or differences were assessed by one-way ANOVA using the Statistical Package for Social Science, version 18.0 (SPSS, Chicago, IL). Linear regression models were used in multivariate analyses. *P* values <0.05 were considered significant.

Results

NNT mRNA expression was detectable both in subcutaneous and visceral fat depots without gender specific differences for either subcutaneous or visceral *NNT* gene expression (data not

shown). *NNT* gene expression in subcutaneous and visceral adipose tissue was comparable (Figure 1A). To exclude a potential effect of impaired glucose metabolism on *NNT* mRNA expression, we selected a healthy subgroup of lean (BMI <25 kg/m^2) men (N = 19) and women (N = 22) with normal glucose tolerance (NGT). Likewise, *NNT* mRNA expression was equal in visceral and subcutaneous adipose tissue (Figure 1B). Comparison of *NNT* mRNA expression in subgroups of BMI <25 or >30kg/m² revealed significantly higher visceral *NNT* mRNA expression in the obese subgroup (Figure 1C). In contrast to visceral *NNT* expression, sc *NNT* mRNA expression was not significantly different between BMI subgroups (Figure 1D). We found a significant correlation between subcutaneous and visceral *NNT* gene expression (Figure 1E; r = 0.448, P = 0.003). Furthermore, visceral *NNT* mRNA expression was not different between the normal control and type 2 diabetes subgroup (Figure 1F).

Univariate correlation analyses of the entire study population identified significant correlations between visceral (but not with subcutaneous) *NNT* gene expression and BMI (r=0.195; p=0.003), % body fat (r=0.183; p=0.025), waist and hip circumferences (waist: r=0.220; p=0.001; hip: r=0.183; p=0.006), sc and visceral fat area (sc: r=0.222; p=0.002; vis: r=0.215; p=0.003) as well as fasting plasma insulin (r=0.157; p=0.038) and leptin serum concentrations (r=0.205; p=0.008) (Table 2). There was no correlation between *NNT* gene expression and age, lipids, HbA1c and fasting plasma glucose (Table 2). After adjusting for age, gender and BMI, only % body fat (r=0.191; p=0.032) and circulating leptin (r=0.260; p=0.016) were significantly associated with visceral *NNT* mRNA expression (Table 2). We further tested the hypothesis that visceral *NNT* expression may predict parameters of obesity, fat distribution, insulin sensitivity, and glucose tolerance by multivariate linear regression analyses (Table 3). These analyses identified visceral *NNT* expression as age and gender independent predictor of BMI (r=0.174; p=0.003), waist circumference (r=0.181; p=0.002), visceral fat area (r=0.194; p=0.005) and % body fat (r=0.145; p=0.032), but not fasting

plasma insulin and 2h OGTT glucose (Table 3). However, the significant associations between visceral *NNT* expression and parameters of fat distribution and glucose metabolism are not independent of the significant association with BMI (Table 3).

Discussion

In male B6J mice, a mutation in the *Nnt* gene is associated with reduced glucose-stimulated insulin secretion *in vitro,* impaired glucose tolerance, higher epigonadal fat mass and higher susceptibility to diet induced obesity. Until now, a potential role of NNT in human obesity has not been established. Human genetic data obtained from a genetic association database (http://geneticassociationdb.nih.gov) and GWAS using BMI and waist circumference as traits (http://www.gwascentral.org/generegion/phenotypes) do not suggest genetic variation within NNT being a major player in the pathophysiology of obesity or insulin resistance. Here we demonstrate that *NNT* expression in human visceral adipose tissue is associated with parameters of obesity and fat distribution and may play a role in the development of obesity. We found a significant correlation between subcutaneous and visceral NNT gene expression, but only visceral *NNT* mRNA levels differed between lean and obese subjects. In addition, only visceral NNT expression correlates with parameters of obesity, fat distribution and glucose metabolism, suggesting that in the absence of genetic determinants, visceral *NNT* mRNA may increase with increasing fat accumulation and visceral fat distribution. The lack of associations between sc NNT expression and BMI or measures of fat distribution despite a correlation between visceral and sc NNT expression maybe due to a disproportionally higher increase in visceral compared to sc adipose tissue NNT expression with increasing BMI. Such divergent relationships between visceral and sc adipose tissue have been found for several parameters in insulin sensitive compared to insulin resistant obese individuals independently of BMI or body fat mass (17). Higher visceral NNT expression in (visceral) obesity may in

addition reflect previously reported predominant development of adipose tissue dysfunction in visceral compared to subcutaneous depots (18-19).

Based on our data, we are not able to establish a cause-effect relationship similar to the associations in B6J mice. Additional NNT genotype - phenotype association studies could clarify whether genetic variants in the *NNT* gene are related to obesity and fat distribution in humans.

It has been hypothesized that reduced Nnt expression affects ATP production, thus impairing ATP-sensitive potassium channel activity consequently leading to impaired glucose stimulated insulin release in isolated islets and MIN6 cells (4, 13). Increasing native Nnt levels in B6J mice by transgenic overexpression rescued impaired insulin secretion (12). However, high Nnt expression seems to represent a double-edged sword, as backcross hybrids of C57BL/6J and diabetes susceptible DBA/2 mice revealed that high Nnt expression and activity in DBA/2 mice correlated with insulin hypersecretion indicating a predisposition of the DBA/2 strain to early beta cell failure and future development of type 2 diabetes (11). Moreover, Anderson et al. showed, that fourfold higher Nnt expression in islets of C57BLKS/J (BLKS) mice which do not harbour a mutant Nnt gene may significantly contribute to increased insulin secretion (20). These differences in Nnt expression may underly the higher susceptibtibilty of BLKS mice to islet exhaustion in insulin-resistant states as compared to C57BL6/J mice (20). A recent study showed that Nnt has little influence on insulin secretion under normal or low Nnt expression levels *in vitro* (21). They further showed that very likely other enzymes are able to compensate for reduced $NADP^+$ reduction activity compared to normal Nnt expression levels (21). *In vivo* insulin secretion and glucose tolerance, glucose-mediated insulin secretion and insulin sensitivity were comparable between B6N and B6J mice (21). Furthermore, they could show that high level expression of native Nnt in MIN6 cells could significantly affect and enhance insulin secretion, which is in agreement with the rescue effect demonstrated for transgenic Nnt overexpression in B6J mice

and supports the suggested mechanism of insulin hypersecretion in DBA/2 mice (11-12, 21). Here, we did not investigate *NNT* expression in islets, thus correlation between adipose tissue *NNT* expression and increased plasma insulin concentrations does not establish a causative role of NNT in the regulation of insulin secretion. We found *NNT* expression primarily related to parameters of obesity and fat distribution. Univariate correlation between *NNT* expression and fasting plasma insulin (FPI) did not remain significant after adjusting for age, gender and BMI. Multivariate regression analyses further revealed that associations between visceral adipose tissue *NNT* expression and parameters of glucose metabolism including FPI are not independent of the significant association with BMI.

It has been proposed that *Nnt* mutation and resulting lower levels of the active protein in male B6J mice is associated with higher epigonadal fat mass and higher susceptibility to diet induced obesity. Thus, it could be speculated, that higher expression of NNT in adipose tissue could be beneficial for protection against abdominal fat accumulation and subsequently for whole body glucose metabolism. In PC12 rat pheochromocytoma cells, siRNA induced Nnt suppression resulted in increased oxidative stress with subsequent impairment of mitochondrial function, initiation of apoptosis resulting in decreased cell viability (22). Translocation of redox-sensitive c-Jun N-terminal kinase (JNK) to the mitochondrion due to decreased GSH/GSSG ratios and increased H_2O_2 levels have been proposed as underlying mechanisms (22). Therefore, higher NNT expression in adipose tissue could be protective against adipose tissue dysfunction via regulation of redox-sensitive signaling by H_2O_2 and prevention of mitochondrion dependent intrinsic apoptosis. Increased visceral NNT expression could therefore represent a compensatory mechanism to protect adipose tissue against adipose tissue dysfunction associated with increasing fat mass and BMI.

The associations between altered *Nnt* expression in B6J mice and parameters of glucose metabolism may therefore be secondary to the relationship between NNT and obesity. Using our human data set, we aimed to dissect the associations between *NNT* expression and obesity

parameters from those between *NNT* and parameters of glucose metabolism. Interestingly, we found that *NNT* expression is primarily related to parameters of obesity and fat distribution whereas associations between *NNT* and fasting plasma insulin concentration or 2h OGTT glucose are not significant beyond the relationship with BMI or body fat mass. Therefore, our data suggest a role of *NNT* in the development of obesity and fat distribution, which may underlie the association between NNT and impaired glucose metabolism in B6J mice.

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Disclosure

The authors declared no conflict of interest.

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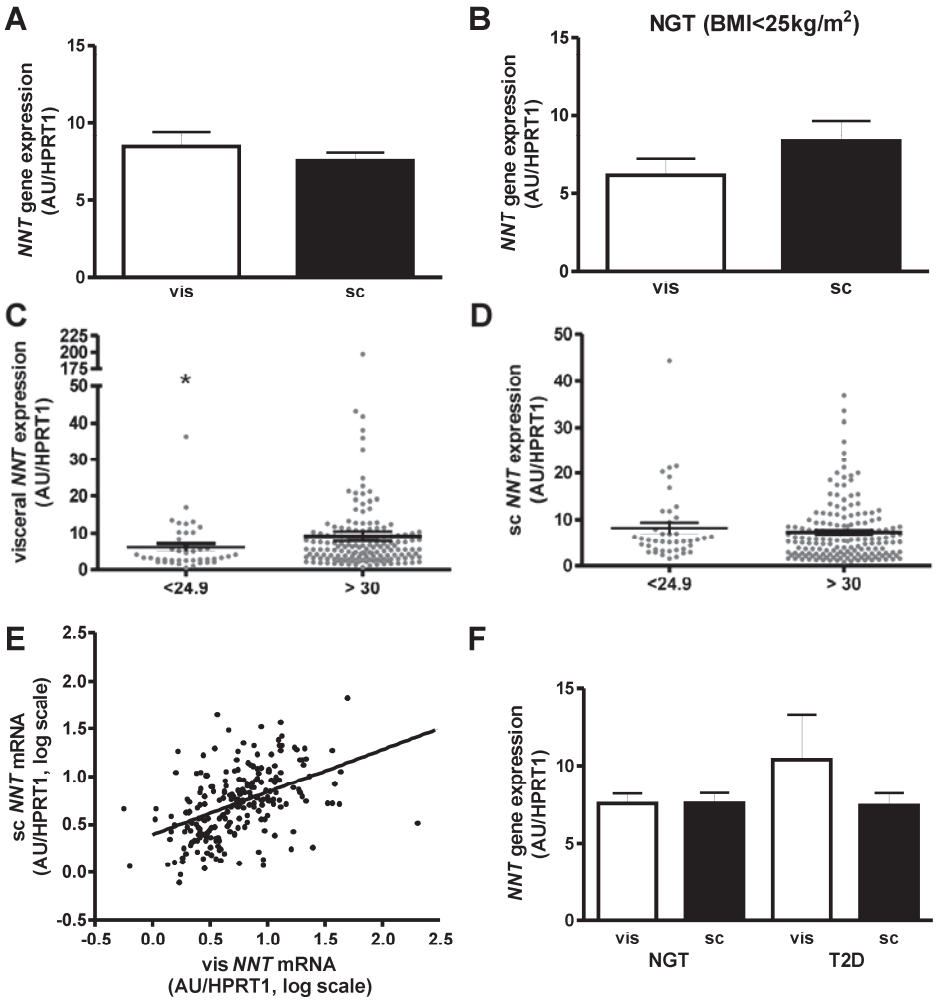

Fig.1. ***NNT*** **mRNA expression in human paired samples of visceral (vis) and subcutaneous (sc) adipose tissue**. Expression in the entire Caucasian cohort (N=221) (A) and in a subgroup of lean, normal glucose tolerant subjects (NGT) (BMI <25 kg/m^2; N=38 (vis), 38 (sc)) (B). Visceral (C) and subcutaneous (D) *NNT* mRNA levels in BMI subgroups (lean: BMI <24.9 kg/m^2; N= 42 (vis), 42 (sc) and obese: BMI >30 kg/m^2; N=159 (vis), 163 (sc)) and correlation analysis between sc and visceral *NNT* mRNA levels (E). Data were log transformed to achieve normal distribution. Fat depot specific *NNT* expression levels in type 2 diabetic patients (N=69 paired samples) in comparison with healthy NGT subjects (N=152 paired samples) (F). Data are presented as means ± SEM. **P* < 0.05 between groups.