THE NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE GENE COULD IMPAIR BLOOD GLUCOSE LEVEL STABILITY AND INCREASE BASAL METABOLISM

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C57BL/6J (B6J) is the most widely used mouse strain for metabolic research. It carries a spontaneous mutation in the nicotinamide nucleotide transhydrogenase (Nnt) gene. This study compared blood glucose levels in B6J and Kunning (Km) mice as control. It was observed that blood glucose levels in B6J and Km mice at 4 weeks of age decreased post feeding and reached the lowest level at 24 hr of fasting. Blood glucose was significantly higher in B6J mice than that in Km mice at 0 hr, 2 hr and 10 hr of fasting. In addition, the correlation between the Nnt gene, growth traits and the feed conversion efficiency ratio (FCR) in (B6J × Km) F2 generation (N = 342) was also analyzed. We found that Nnt was significantly associated with body weight at 3 weeks (IBW) (P<0.01) and 5 weeks (FBW) (P<0.05) and average metabolic body weight (AMBW) (P<0.01) but was not associated with average daily feed intake (AFI), average daily gain (ADG) and FCR. Furthermore, qRT-PCR revealed that the expression levels of Glut-1, Glut-2, Akt-1, Irs-1 and Ucp-2 genes were significantly different in high and low-FCR mice. Our study offers novel evidence of the roles of Nnt gene in metabolism and growth.

Keywords: Nnt, blood glucose, familial glucocorticoid deficiency, glucose metabolism, autosomal recessive disorder, feed conversion ratio, mice

INTRODUCTION

Nicotinamide nucleotide transhydrogenase is located at the inner membrane of the mitochondria and catalyses the reversible reduction of NADP⁺ by NADH (Adedeji, 2009; Kelsey et al., 2015) which is important for integrating the mitochondrial tricarboxylic acid cycle (TCA) cycle and energy metabolism (Natascia et al., 2006; Fei et al., 2012; Andrikopoulos, 2010). Several studies have indicated that the Nnt gene is significantly associated with human disease such as obesity (Toyoe et al., 2005; John et al., 2013) and Type 2 Diabetes (T2D) (Helen et al., 2006; Grace et al., 2014; Sadie and Nair, 2010). While, a few studies recently have reported that Nnt gene is related to familial glucocorticoid deficiency (FGD) which is a rare autosomal recessive disorder (Yasuko et al., 2015; Juliana et al., 2013; Eirini et al., 2012).

Previously, several studies have also been proposed that Nnt gene could participate in glucose metabolism (Helen et al., 2006). A five exon deletion of Nnt gene in B6J mice, which exhibits impaired glucose tolerance (IGT) and reduced insulin secretion, has been identified (Sofianos et al., 2005). Furthermore, QTL mapping results indicated that the Nnt gene could be a candidate gene for glucose metabolism (Brand et al., 2010). However, other studies indicate that mutating Nnt is associated with impaired β-cell mitochondrial metabolism, possibly via activation of uncoupling proteins, which may regulate glucose-stimulated insulin secretion (GSIS), energy balance, body weight and thermoregulation (Neil et al., 2010; Adedeji et al., 2009; Jessica and Carter-Su, 1988).

Most cells utilize glucose as their primary fuel source for energy (Jonhan et al., 2012). Glucose uptake typically is mediated by a family of facilitative glucose transporters (GLUTs 1–4) that is differently regulated, reflecting their specific roles in cellular and whole body glucose homeostasis (Luc, 2013; Douglas et al. and Fan, 2009). It has been reported that glucose metabolism is related to growth (Anthony et al., 2010). Yamada and co-workers study indicates that the fasting levels of glucose were negatively correlated with plasma growth hormone (GH) levels (Yamada et al., 2010) which can significantly stimulate the
glucose oxidation (Møller et al., 1990). Moreover, insulin-like growth factor I and II (IGF-I/II) could also stimulate an increase in glucose oxidation, with a longer and stronger effect than GH (Philip et al., 1997). Further, thymoma viral proto-oncogene 1 (Akt-1) plays an important role in mediating glucose metabolism and protein synthesis (Bhanu et al., 2010). Furthermore, Akt-1 has a distinct role in glucose metabolism, maintaining insulin sensitivity and modulating the expression of the insulin receptor substrate-1 expression (Irs-1) gene (Hana et al., 2012).

It has been reported that energy metabolism is related to feed efficiency (FE), which is a complex trait in animals (McDonald et al., 2009). FE can be measured by a feed conversion ratio (FCR) or residual feed intake (RFI) (Chen et al., 2011). Our previous study indicated that mitochondrial energy metabolism was negatively related to FE in pigs (Lu et al., 2015). Another study indicated that carbohydrate metabolism has also been related to FE in cattle (Chen et al., 2011). Although the Nnt gene plays a role in glucose metabolism, the role of Nnt in FE is unclear. To investigate the role of Nnt gene in glucose metabolism, the blood glucose level of Km mice to normal Nnt and B6J mice with a mutant Nnt were compared. Moreover, the blood glucose levels between F2 generations (B6J × Km) of mice having different Nnt genotypes were also evaluated. Further, the roles of Nnt gene in growth and FE were detected by trait association studies in F2 generation mice. Furthermore, expression analysis of the genes related to glucose metabolism in liver tissues was also performed.

**MATERIALS AND METHODS**

**Experimental animals:** B6J and Km mice were obtained from the Wuhan University Center for Animal Experiments and the Hubei Centre for Disease Control and Prevention at 3-5 weeks of age. Mice were housed under controlled temperature (21±2°C) on a 12:12 hour light- dark cycle with free access to food and water. For F2 generation, seven Km males were crossed with seven B6J females to generate 84 F1 mice which were crossed to generate F2 mice. Then, 342 F2 generation mice were used for the association study of Nnt gene. All the mice were fed a standard diet. The feed intake of the F2 mice at 3 to 5 weeks of age was measured. Additionally, the body weight at 3 and 5 weeks of age was measured (IBW and FBW). Furthermore, ADG, AMBW and FCR were measured as follows:

\[
\text{ADG} = \frac{\text{FBW} - \text{IBW}}{14}; \quad \text{AMBW} = \left[\frac{\text{IBW} + \text{FBW}}{2}\right]^{0.75}; \\
\text{FCR} = \frac{\text{AFI}}{\text{ADG}}.
\]

All the methods in this study were carried out in accordance with the approved guidelines from the Regulation of the Standing Committee of Hubei People’s Congress. Furthermore, all experimental protocols were approved by the Ethics Committee of Huazhong Agricultural University (HZAUMU2013-0005).

**Blood glucose measurement:** The blood glucose level was detected using a blood glucose monitor (Andon, Tianjin, China). For blood glucose level detection, 11 B6J and 11 Km mice at four weeks of age were selected randomly. In addition, 40 mice of the F2 generation at five weeks of age with extremely high and low-FCR were selected for blood glucose level detection. All the mice used for blood glucose level detection were fasted overnight (12 hours), and then fed ad libitum for one hour. Subsequently, the blood glucose levels were detected at 2 hr, 6 hr, 10 hr, 14 hr and 24 hr of fasting.

**RNA extraction and cDNA synthesis:** The total RNA was extracted from liver tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Fermentas, Ottawa, ON, Canada). The concentration and quality of the RNA was assessed using NanoDrop 2000 (Thermo, Waltham, MA, USA). Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific Inc.).

**PCR and Quantitative Real Time-PCR (qRT-PCR) assays:** To identify the Nnt mutation, we designed two pairs of primers for cDNA amplification. The first primers (Nnt-D1) could amplify cDNA of both the mutant and the normal Nnt gene; the second primers (Nnt-D2) could only amplify the normal gene. All the sequences of the primers used in this study are listed in Table 1. For genotyping of the F2 generation, two pairs of primers were used. The first pair of primers (Nnt-Km) could only amplify Nnt DNA of Km mice (Stefanie et al., 2012); the second pair of primers (Nnt-B6J) could only amplify Nnt DNA of B6J mice.

The expression levels of Glut-1, Glut-2, Glut-4, Ucp-2, Akt-1, Irs-1, Igsf-1 and Igf-1 were detected by qRT-PCR and the primers are also listed in Supplementary Table 1. The qRT-PCR was performed by using iTaq™ Universal SYBR® Green Supermix (Bio Rad) and the CFX96 machine (California, USA).

**Statistical analysis:** For the association study, we used the SAS 8.1 statistical package to perform a least-squares analysis (Russell, 1997). The model adopted is as follows:

\[
Y = X\beta + Z\tau + \epsilon,
\]

where Y is the phenotypic trait value for IBW, FBW, AFI, ADG, AMBW and FCR, respectively; X\beta is the genotype effect of Nnt gene. AA, AB and BB indicate Nnt genotypes Nnt −/−, Nnt +/− and Nnt +/+; respectively. Z\tau is the random effect of gender; and \epsilon is the random error.

For the difference analysis of blood glucose level and gene expression, we used Student’s T- test (two- tailed distribution, two samples and unequal variances) or F- test (one- way ANOVA) and the levels of statistical significance were determined at P < 0.05 and P < 0.01.

**RESULTS**
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Nnt mutation detection: To confirm the deletion mutation of the Nnt gene, two pairs of primers were used for PCR. The first pairs of primers generated a 435 bp PCR product in the B6J mice and a 1189 bp PCR product in the Km mice (Fig. 1a, b). The second pairs of primers were designed from exon 6 to exon 7 so that only the normal Nnt could be amplified. Using these primers, there was a 216 bp product in the Km mice and no PCR product in the B6J mice (Fig. 1c).

Table 1. The sequence of primers used.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>PCR product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nnt-D1</td>
<td>F- AGCTCAGGGCTATGATGTC</td>
<td>62°C</td>
<td>B6J: 435 bp</td>
</tr>
<tr>
<td></td>
<td>R- TCTGGAGGCTCCGTGGTGC</td>
<td></td>
<td>Km: 1189 bp</td>
</tr>
<tr>
<td>Nnt-D2</td>
<td>F- CTTGCTGCTGACCCCTTGG</td>
<td>60°C</td>
<td>B6J: – bp</td>
</tr>
<tr>
<td></td>
<td>R- TGACCTTCCTCTCAGGTACTC</td>
<td></td>
<td>Km: 216 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F- GGGTGTGAGACAAGAGAAAAT</td>
<td>60°C</td>
<td>200 bp</td>
</tr>
<tr>
<td></td>
<td>R- CTTTCCCAATAGCACAAGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nnt-Km</td>
<td>F- GGGCATAGGAGCAATAACAGTTG</td>
<td>59°C</td>
<td>549 bp</td>
</tr>
<tr>
<td></td>
<td>R- GTAGGGCCACTGTTCTGCAATGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nnt-B6J</td>
<td>F- GCCAGAAGAGTTTATCATTAGGGAGACCCGC</td>
<td>60°C</td>
<td>522 bp</td>
</tr>
<tr>
<td></td>
<td>R- GTATCAACAGTTTATGGGACACCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glut-1</td>
<td>F- CTTAGACATACATGGGAGACCCGC</td>
<td>58°C</td>
<td>175 bp</td>
</tr>
<tr>
<td></td>
<td>R- TTGGAGGAAACCCATAAACGACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glut-2</td>
<td>F- GGTCCCTCAGTCTCCTGTGCTGTA</td>
<td>61°C</td>
<td>131 bp</td>
</tr>
<tr>
<td></td>
<td>R- AGCTCATGTTAATGGCGACTTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glut-4</td>
<td>F- ATGGAATGCTGCTGCTGCTCCTCT</td>
<td>60°C</td>
<td>132 bp</td>
</tr>
<tr>
<td></td>
<td>R- TATGGTGCGTAGCTGCTGCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ucp-2</td>
<td>F- GGAAGTACAGGAAATCAGA</td>
<td>61°C</td>
<td>190 bp</td>
</tr>
<tr>
<td></td>
<td>R- TAGGAAGGTGATTACGAAGGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akt-1</td>
<td>F- TACTCCTCTCTCAAGAAGATGGCA</td>
<td>61°C</td>
<td>160 bp</td>
</tr>
<tr>
<td></td>
<td>R- ACATGGAAGGTGCGCTCAATGACTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irs-1</td>
<td>F- AGAAGAGCCAGGACATGCTCAATA</td>
<td>60°C</td>
<td>120 bp</td>
</tr>
<tr>
<td></td>
<td>R- AGTCGGCCACTGCTAGGAGGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Igf-1</td>
<td>F- CCAACTCATTATATGGAAGCTGCC</td>
<td>60°C</td>
<td>141 bp</td>
</tr>
<tr>
<td></td>
<td>R- TCCACACGAGAAGAACGACATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td>F- GACTATGAGACTCGTGTGGCTC</td>
<td>60°C</td>
<td>150 bp</td>
</tr>
<tr>
<td></td>
<td>R- TATTTCTCTCCGAGATGTGGC</td>
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</table>

Blood glucose levels were different between B6J and Km mice: To confirm the relationship between Nnt and glucose levels, we compared the blood glucose variation in Km and B6J mice in the fasting state. All mice were firstly fasted overnight (12 hours) and then fed one hour, which was defined as 0 hours of fasting. The glucose levels at 6 times points were detected; 0 hours, 2 hours, 6 hours, 10 hours, 14 hours and 24 hours of fasting of B6J and Km mice. The blood glucose levels declined regularly post feeding and reached the lowest level at 24 hr of fasting in both strains (Table 2).

Table 2. Blood glucose concentration (mmol/L) at 4 weeks of age in the B6J and Km mice.

<table>
<thead>
<tr>
<th>Fasting Time (hours)</th>
<th>B6J mean±SE (n = 11)</th>
<th>Km mean±SE (n = 11)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>16.70±0.73</td>
<td>11.71±0.41</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>2h</td>
<td>13.06±0.47</td>
<td>10.97±0.36</td>
<td>0.02</td>
</tr>
<tr>
<td>6h</td>
<td>10.76±0.32</td>
<td>10.40±0.49</td>
<td>0.54</td>
</tr>
<tr>
<td>10h</td>
<td>10.37±0.62</td>
<td>8.75±0.26</td>
<td>0.03</td>
</tr>
<tr>
<td>14h</td>
<td>8.09±0.37</td>
<td>8.03±0.32</td>
<td>0.90</td>
</tr>
<tr>
<td>24h</td>
<td>4.72±0.09</td>
<td>6.95±0.28</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

Moreover, the blood glucose levels of B6J mice at fasting, 0 hr, 2 hr and 10 hr were significantly higher in Km mice than in B6J mice. At 24 hr of fasting, the blood glucose level was significantly higher in Km mice than in B6J mice. No significant
differences were observed at the other time points. Furthermore, we used F1 generation (B6J × Km) to construct F2 generation (N = 342). For the glucose detection, the extremely high (N = 20) and low (N = 20) FCR mice (P<0.01) were selected (Table 3). However, no significant differences were observed at 0 hr, 12 hr and 24 hr of fasting in male and female mice.

**Association analysis of the Nnt with growth and FE traits:** To analyse the role of Nnt in growth and feed efficiency traits, we studied the correlation between glucose metabolism and FE, we detected the expression genes related to glucose metabolism in the liver tissue of the high and low FCR mice. The expression levels of Glut-1, Glut-2, Glut-4, Ucp-2, Akt-1, Irs-1 and Igf-1. Glut-1 and Ucp-2 expression levels were higher in the high-FCR mice than in the low-FCR mice. The expression levels of Akt-1, Glut-2 and Irs-1 genes were higher in the low-FCR mice than in the high-FCR mice. Moreover, the IBW, FBW and AMBW values for the AA (Nnt −/−) and AB (Nnt +/−) genotypes were significantly higher than for the BB (Nnt +/+ ) genotype (P<0.05).

**Expression of other glucose metabolism related genes in the liver tissue of the high and low-FCR mice:** To further investigate the correlation between glucose metabolism and the FCR trait, we also detected the expression levels of Glut-1, Glut-2, Glut-4, Ucp-2, Akt-1, Irs-1 and Igf-1. Glut-1 and Ucp-2 expression levels were higher in the high-FCR mice than in the low-FCR mice. The expression levels of Akt-1, Glut-2 and Irs-1 genes were higher in the low-FCR mice than in the high-FCR mice. Because the Nnt gene was not significantly associated with Glut-4 and Igf-1 genes expression.

**DISCUSSION**

NNT is considered a high-capacity source of mitochondrial
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NADPH, which is located at the inner mitochondrial membrane (Yu-Ting et al., 2012). In this study, we first confirmed that there was a deletion mutant of Nnt in B6J mice of 754 bp in the exon. Some studies indicated that the mutant Nnt could affect glucose metabolism (Freeman et al., 2006). However, another study reported that there was no correlation between Nnt and glucose metabolism (Josef et al., 2002). Therefore, the role of Nnt in glucose metabolism is not clearly understood.

To study the role of the Nnt gene in glucose metabolism, we compared the blood glucose level variations in the Nnt mutant B6J mice and the Nnt normal Km mice. Also, we analyzed the blood glucose level variations in the (B6J × Km) F2 generation. According to our results, post feeding, the blood glucose level reached a very high level and then gradually declined until 24 hr of fasting. In addition, the blood glucose level of the B6J mice was higher than the Km mice at the early stage of fasting, whereas it was lower than that in the Km mice at 24 hr. These results indicate that the glucose level of the Km mice was metabolized more slowly than in B6J. Also, the blood glucose level variation of Nnt−/− genotype was significantly higher than that of Nnt+/+ genotype in the F2 generation. These results indicate that the Nnt gene could participate in the glucose metabolism and may maintain the stability of the glucose level. Nnt also plays a critical role in energy metabolism. The ATP level was reduced when the Nnt gene was knocked down (Fei et al., 2012). Many studies have suggested that energy metabolism is related to FE (Chen et al., 2011). The role of Nnt in FE is not clear. In this study, we found that Nnt was associated with AMBW and body weight, but not with FE. AMBW is positively correlated with basal energy metabolism (Chan et al., 2001; Stefano and Mammucari, 2011).

Further, to investigate the differences in the body weight (BW) and growth between B6 and Km mice, the body weights of mice were measured at 3 weeks and 5 weeks of age. We observed that the body weight and growth of B6J and Km mice were significant different at 5 weeks age (p < 0.01) (Fig. 5). While, there were no significant differences at 3 weeks of age (p = 0.2691). Moreover, a previous study confirmed that mice carrFeig a mutant Nnt gene grew faster when fed a high-fat diet (Emily et al., 2013). Therefore, Nnt gene was associated with the body weight, indicating that Nnt gene may play a role in growth processes.

Importantly, the glucose transporter genes were significantly expressed higher in the high-RFI than that in the low-RFI in the livers of Angus cattle (Polyana et al., 2015). Further, one study has reported that the expression of glucose transporter genes is variant between distinct species, tissues and cellular subtypes and each has differential sensitivities to stimuli such as insulin (Sumera et al., 2012). Our results showed that, the blood glucose level was not significantly different between the high and low-FCR mice. In addition, the Glut-4 gene was not significantly different between the high and low-FCR mice. Although the Glut-1 and Glut-2 genes were significant, their expression patterns between high and low-FCR mice were the opposite. Therefore, our results are consistent with a previous study showing that the expression level of Glut-1 gene was higher than that of Glut-2 and Glut-4 in the liver tissue of rats (Michael et al., 1991; Cedric et al., 2013). Based on these results, we demonstrated that the blood glucose level may not associate with FE in mice.

Additionally, the expression level of Igf-1 gene was not significantly different between the high and low-FCR mice. Further, we found that the expression of Akt-1 and Irs-1 genes was significantly higher in the low-FCR mice. Akt-1 and Irs-1 genes are key genes of the IGF signaling pathway (Chan et al., 2001), which plays an important role in muscle growth and glucose metabolism (Stefano and Mammucari, 2011; Neil and Johnston, 2010). Therefore, we concluded that muscle growth might positively relate to FE in mice. It has been reported that the expression of Ucp-2 is positively related to energy expenditure (Jessica and Carter-Su, 1988). According our results, the Ucp-2 was significantly higher in the high-FCR mice. Therefore, we deduced that energy expenditure was negatively related to FE in mice.

Conclusion: The blood glucose level of mutant Nnt B6J mice was significantly different than normal Nnt Km mice. The blood glucose level of the Km mice was metabolized more slowly than in B6J mice under fasting conditions. Additionally, the Nnt gene was associated with body weight and AMBW, but was not associated with FE. The expression levels of the Akt-1 and Irs-1 genes were higher and Ucp-2 gene expression was lower in the liver of the high-FCR mice. Therefore, the Nnt gene could play a role in glucose metabolism and basal energy metabolism in mice.

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REFERENCES


The role of Nnt gene in blood glucose level & basal metabolism


Nicole, W., B.R. Amy, M. Grant and A. Sofianos. 2010. The deletion variant of Nicotinamide Nucleotide Transhydrogenase (Nnt) does not affect insulin secretion or glucose tolerance. Endocrinology 151:96-102.


