Overexpression of TRB3 Gene in Adipose Tissue of Rats with High Fructose-induced Metabolic Syndrome

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Abstract. Insulin resistance is the physiopathologic foundation of metabolic syndrome. TRB3 has been revealed to be involved in insulin resistance in the liver by interacting directly with Akt and blocking its activation. Our investigation aims at exploring the relationship between metabolic syndrome and TRB3 mRNA expression in adipose tissue of rats. Two groups were studied as follows: the control group (Control, n = 12) was fed a standard rodent chow, and the experimental group (Fructose, n = 9) was fed a high-fructose diet. Body weight and systolic blood pressure were measured per 4 weeks. At the end of 38 weeks, levels of tribbles mRNAs in adipose tissue were determined by quantitative real-time polymerase chain reaction (PCR), and Akt/phospho-Akt expression was assessed by Western blot. Results show that levels of TRB1-3 mRNAs were expressed in adipose tissue of rats of both groups, and tribbles mRNAs were TRB1 (Control: 0.00515, Fructose: 0.00497), TRB2 (Control: 0.02104, Fructose: 0.01988), and TRB3 (Control: 0.00457, Fructose: 0.00822), respectively. Of the three, TRB3 mRNA alone significantly increased by 94% in adipose tissue of fructose-fed rats compared with those in adipose tissue of the controls (P<0.05), and there was significant positive correlation between TRB3 mRNA levels and HOMA-R in fructose group (r = 0.68, P<0.05). Western blot analysis showed that phospho-Akt (Ser-473) expression was significantly decreased in adipose tissue of fructose-fed rats compared with controls (P<0.001). The present study suggests that TRB3 may be involved in metabolic syndrome by inhibiting activation of Akt in adipose tissue.

Key words: Metabolic syndrome, TRB3, Adipose tissue, Insulin resistance, Akt

THE metabolic syndrome is a clustering of risk factors known to increase the risk for the development of diabetes mellitus and cardiovascular disease [1], but the underlying mechanism is not known. Insulin resistance is implicated as a hallmark and physiopathologic foundation of metabolic syndrome, but there are still different opinions for the reason. Therefore key gene resulting in insulin resistance should be identified for the amelioration of metabolic syndrome through the gene intervention.

TRB3 (Drosophila Tribbles-homologue proteins) belongs to a newly identified family of proteins that consists of three known mammalian members: TRB1, TRB2, and TRB3 [2]. Much interest has been recently focused on TRB3, which is involved in the pathophysiology of insulin resistance as a negative modulator of Akt. Du et al. reported that amounts of TRB3 mRNA and protein were significantly increased in livers of db/db diabetic mice compared with those in wild-type mice. Hepatic overexpression of TRB3 in amounts comparable to those in db/db mice promoted hyperglycemia and glucose intolerance [3]. TRB3 (Q84R), which has higher capacity of inhibiting Akt activation, is found to be associated with insulin resistance and related cardiovascular risk [4]. These findings reveal that TRB3 plays a crucial role in the development of
insulin resistance, and liver is often investigated as target tissue.

Adipose tissue is also considered as dominant sites affecting systemic insulin resistance, and especially visceral adipose tissue plays an important role in metabolic syndrome. Adipose tissue secretes several hormones, such as leptin and adiponectin, and a variety of adipocytokines (IL-1beta, IL-6, IL-8, IL-10, TNF-alpha, TGF-beta), which are involved in the development of metabolic syndrome [5–7]. Tribbles (TRBs) genes are not only expressed in liver, thymus gland, pancreatic gland etc., but also overexpressed in multiple tumor tissues [8]. However, the quantitative expression of TRBs in adipose tissue remains unclear, and studies in vivo on the correlation between TRB3 and metabolic syndrome are often restricted in genetic rodent models [3, 9].

To check the role of TRB3 in the development of metabolic syndrome via adipose tissue, the gene expression of TRBs were investigated in adipose tissue of rats fed a standard and a high-fructose diet respectively by using quantitative real-time polymerase chain reaction (PCR), and Akt/phospho-Akt expression was assessed by Western blot.

Materials and Methods

Animal model

All procedures were performed according to institutional guidelines for animal experimentation. The 21 male Wistar rats with body weight range of 140–160 g were obtained from the Central Animal House, Medical College, Shandong University. They were housed in a room under conditions of controlled temperature (20–25°C), humidity (50%–60%), and a 12 h light/dark cycle; and fed a standard rodent chow (provided by Qilu Hospital Animal House) and water ad libitum. After an adaptation of one week the animals were divided randomly into two groups: a control group (Control, n = 12) in which the rats were fed a standard rodent chow and water, and a high-fructose diet group (Fructose n = 9) in which the rats were fed the standard rodent chow plus fructose in the drinking water as a 10% (w/v) solution. The rats had free access to food and water during the experiment for 38 weeks.

Body Weight and Systolic blood pressure (BP) Measurement

At the beginning of experiment, body weight and systolic BP were measured, and this was repeated per 4 weeks thereafter. Systolic BP was monitored for 5 times in conscious, prewarmed, slightly restrained rats by the tail-cuff method [10] described by Kojima, and average value was adopted.

Blood and Tissue Sampling

At the end of 38 weeks, the rats were deprived of food for 12 h. Then the blood was collected from jugular vein, and plasma was obtained from the blood using a centrifuge (2000 g) at 4°C for 20 min. All animals were sacrificed by cervical decapitation. Finally the epididymal fat pads were excised, frozen in liquid nitrogen and stored at −80°C before processing.

Biochemistry Determinations

Total cholesterol (TC), triglyceride (TG) and blood glucose (Glu) were determined using enzymatic photolorimetry with a test kit purchased from the Audit Diagnostics Company (Cork, Ireland). The plasma insulin was determined using radioimmunoassay (RIA). The R-value of homeostasis model (HOMA-R), which was calculated by the formula HOMA-R = fasting glucose (mmol/l) * fasting insulin (μU/ml)/22.5 [11], was expressed as an index of insulin resistance.

Quantitative Real-Time PCR

The epididymal fat pads were homogenized in 1 ml Trizol reagent with the use of a glass-on-glass homogenizer, and total RNA was isolated following the manufacturers’ instructions. The purified RNA was treated with DNase I (DNase-free; Ambion, Austin, TX) for 30 min at 37°C to remove DNA and then reverse transcribed with a reverse transcription system (Promega, Madison, WI). Real-time PCR was performed with DyNAmo SYBR Green I Master Mix reagent (TaKaRa Bio Group, Japan) in the DNA Engine Opticon System (MJ Research, Reno, NV). Table 1 shows the specific primers used for the synthesis and amplification of the cDNA of interest. Fluorescence was monitored during PCR by the Opticon Fluorescence Detection System (MJ Research, Reno, NV).
The size of the qPCR products was confirmed by comparing the size of product with the commercial ladder after agarose gel electrophoresis. PCR data were then normalized to GAPDH, and the relative amount of mRNA was determined by calculating $2^{-\Delta Ct}$, where $\Delta Ct$ is the difference of the reaction thresholds cycle (CT) between GAPDH and each of the genes of interest.

**Western Blot Analysis**

Samples were obtained from the epididymal fat pads. Whole-cell protein extraction was performed as described [12]. Equal amounts of the samples were separated by SDS-PAGE and visualized after electrophoretical transfer via binding of specific primary and HRP-conjugated secondary antibodies followed by chemiluminescent detection. The primary antibodies were a 1:300 dilution of the antibody against Akt, a 1:300 dilution of the antibody against phospho-Akt (serine-473) (both from Cell Signaling Technology), and a 1:400 dilution of the antibody against $\beta$-actin (Santa Cruz). Detection and quantification were performed with a Kodak image station (NEN, Cologne, Germany).

**Statistical and Data Analysis**

Data were expressed as mean ± SD. The Fructose group was compared with the Control group, and the statistical significance between two groups was evaluated using the $t$ test. A $p$ value of less than 0.05 was considered statistically significant.

**Results**

**Metabolic Variables**

Chronic administration of fructose as a 10% drinking solution during 38 weeks induced the cluster of metabolic alterations. Rats fed a high-fructose diet had higher body weight than the control group after 20 weeks ($P<0.05$), and at the end of 38 weeks body weight gain was more significant ($P<0.001$). The systolic BP of the Fructose group increased more greatly than the control group after 4 weeks ($P<0.001$), and afterwards kept increasing ($P<0.001$). Three other variables, which include fasting levels of plasma triglycerides ($P<0.05$), plasma insulin ($P<0.001$), and HOMA-R ($P<0.05$) of the Fructose group, significantly increased compared with those of the Control group. There were no significant differences between two groups in total cholesterol and blood glucose in spite of which were slightly increased (Table 2).

**Table 1.** Primers used for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (70 bp)</td>
<td>5’-TGCCAAGTATGATGACATCAAGAAG-3’</td>
<td>5’-AGCCCAAGGATGCCCTTTAGT-3’</td>
</tr>
<tr>
<td>TRB1 (174 bp)</td>
<td>5’-AAATTGTTCGGCGCTGG-3’</td>
<td>5’-GGCAGCCATGTATTCGAC-3’</td>
</tr>
<tr>
<td>TRB2 (150 bp)</td>
<td>5’-TACCTGTTCGCTGCCCTACTCC-3’</td>
<td>5’-CTCCTCTACTGCTCCTAAAA-3’</td>
</tr>
<tr>
<td>TRB3 (259 bp)</td>
<td>5’-TGAGAGGACGAAGTTGTTG-3’</td>
<td>5’-GCAAAGGTCCTACGG-3’</td>
</tr>
</tbody>
</table>

**Table 2.** Metabolic variables in rats at the end of experiment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Fructose</th>
</tr>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>549.10 ± 43.19</td>
<td>654.89 ± 80.49***</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>115.17 ± 4.90</td>
<td>137.95 ± 6.01***</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.81 ± 0.24</td>
<td>1.34 ± 0.74*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>1.66 ± 0.44</td>
<td>1.70 ± 0.50</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.00 ± 0.96</td>
<td>5.59 ± 1.49</td>
</tr>
<tr>
<td>Insulin (uiU/ml)</td>
<td>8.94 ± 2.03</td>
<td>17.34 ± 3.08***</td>
</tr>
<tr>
<td>HOMA-R</td>
<td>2.13 ± 0.66</td>
<td>4.15 ± 1.45*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (Control, n = 12; Fructose, n = 9).

* $P<0.05$, *** $P<0.001$, compared with Control.
TRBs mRNAs expression

Levels of TRB1-3 mRNAs were expressed in adipose tissue of rats of both groups. The mRNA expression levels of TRBs were TRB1 (Control: 0.00515 ± 0.00317, Fructose: 0.00497 ± 0.00220), TRB2 (Control: 0.02104 ± 0.00596, Fructose: 0.01988 ± 0.00788), and TRB3 (Control: 0.00457 ± 0.00250, Fructose: 0.00822 ± 0.00485) respectively. The mRNA expression levels of TRB3 in fructose-fed rats significantly increased by 94% compared with those in control rats (P<0.05), without significant differences in TRB1 and TRB2 mRNAs. Results are expressed as mean ± SD (Control, n = 12; Fructose, n = 9). *P<0.05 vs. control.

Discussion

TRBs, a newly identified family of proteins, were shown to regulate string activity and hence mitosis during ventral furrow formation [2, 13]. All three TRB proteins share a central TRB domain. In addition, each has N-terminal (70–100 residues) and C-terminal (~25 residues) domains, which are neither closely related to any other sequences nor closely related to each other. TRB2 and TRB3 are respectively 41% and 51% identical to TRB1 [14]. The mammalian TRBs, as the member of the protein kinase superfamily [15], all appear to contain the consensus serine/threonine kinase catalytic core, but lack a conserved ATP-binding pocket. TRB1 mRNA was expressed in most human tissues with the highest levels in skeletal muscle, thyroid gland, pancreas, peripheral blood leukocytes, and bone marrow. TRB2 levels were highest in peripheral blood leukocytes, and TRB3 levels were highest in pancreas peripheral blood leukocytes and bone marrow; in addition HeLa cells were found to express TRB1-3 [14].

In our study, the data show that levels of TRB1-3 mRNA expression significantly increased in adipose tissue of rats fed fructose compared with controls (0.89 ± 0.15 vs. 1.32 ± 0.16, P<0.001), and there was no prominent change in Akt expression between two groups (1.37 ± 0.12 vs. 1.56 ± 0.19, P>0.05) (Fig. 3).

Akt Phosphorylation

Western blot analysis showed that phospho-Akt (Ser-473) expression was significantly decreased in adipose tissue of fructose-fed rats compared with controls (0.89 ± 0.15 vs. 1.32 ± 0.16, P<0.001), and there was no prominent change in Akt expression between two groups (1.37 ± 0.12 vs. 1.56 ± 0.19, P>0.05) (Fig. 3).

Fig. 1. TRBs mRNA expression in adipose tissue of rats. Levels of TRB2 mRNA are highest, and levels of TRB1 mRNA are similar to TRB3 mRNAs. Of the three, TRB3 mRNA alone significantly increased by 94% of high-fructose rats compared with those of control rats (P<0.05), without significant differences in TRB1 and TRB2 mRNAs. Results are expressed as mean ± SD (Control, n = 12; Fructose, n = 9). *P<0.05 vs. control.

Fig. 2. The size of TRBs qPCR products. The size of the qPCR products was confirmed by comparing the size of product with the commercial ladder after agarose gel electrophoresis. DNA molecular weight standard reference: 100, 200, 300, 400, and 500 bp. TRB1–3 molecular weight are 174, 150, and 259 bp respectively.
mRNAs were expressed in adipose tissue of rats. Furthermore, levels of TRB2 mRNA are highest, and levels of TRB1 mRNA are similar to those of TRB3 mRNA. The mRNA expression of TRBs in adipose tissue suggests that TRBs may play important roles in metabolism and growth regulation via adipose tissue.

Liver was investigated as target tissue in which TRB3 negatively regulated insulin signaling by interacting directly with Akt and blocking its activation [3, 16, 17]. However, the expression and function of TRB3 in adipose tissue were seldom studied.

Recent research, in which overexpression of TRB3 in adipose tissues resulted in lean mice due to increase fatty acid oxidation [9], demonstrated that TRB3 plays an important role in fat metabolism too. Inconsistent with the notion proposed by Qi and his colleagues [9], we observed that the body weight of TRB3-overexpressing rats increased greatly. Namely, overexpression of TRB3 mRNA in adipose tissues may be associated with obesity, as well as hypertension, hypertriglyceridemia, hyperinsulinemia and insulin resistance (Table 2). On the other hand, our results have much in common with those reported by Du et al. [3]. Amounts of TRB3 RNA are increased 10-fold in livers from db/db diabetic mice compared with those in livers from wild-type mice [3], while our result shows about 2-fold increase in TRB3 mRNA levels in adipose tissue of fructose-fed rats compared with those in adipose tissue of the controls. The discrepancy in amounts of TRB3 mRNA may be attributed to the variance of animal species, modeling method and target tissue. Besides, the data show that there is significant positive correlation between TRB3 mRNA levels and HOMA-R in fructose group, that is, TRB3, but not TRB1 nor TRB2, is involved in metabolic syndrome via adipose tissue.

Our research merely reveals a kind of phenomenon that TRB3 mRNA is overexpressed in adipose tissues of fructose-fed rats. The mechanism whereby TRB3 causes metabolic alterations via adipose tissue remains unclear. As previously described, TRB3 disrupts insulin signaling by binding directly to Akt and blocking activation of the kinase, thereby inducing insulin resistance in liver [3, 16, 17]. Akt, also known as PKB (protein kinase B), is thought to play a key role in insulin signal transduction [18]. Akt is subjected to activation by insulin in a phosphoinositide 3-kinase-dependent manner [19], and the activation of Akt is effected by phosphorylation of Thr-308 and Ser-473. We speculate that the effect of TRB3 on inhibiting Akt activation is also applicable to adipose tissue. In our study, results indicate that phospho-Akt (Ser-473) expression is decreased through long-term administration of fructose, by which phosphorylation of Akt is confirmed to be inhibited in adipose tissue of fructose-fed rats. We accordingly infer that TRB3 may induce insulin resistance by inhibiting activation of Akt in adipose tissue.

In summary, we have described for the first time the gene expression of TRBs in adipose tissue of rats. Furthermore, TRB3 mRNA is overexpressed in adipose tissue of rats with high fructose-induced Metabolic Syndrome, which suggests that TRB3, but not TRB1 nor TRB2, is involved in metabolic syndrome via adipose tissue. Our study also suggests TRB3 may
duce insulin resistance by inhibiting activation of Akt in adipose tissue. TRB3 has become a suitable target molecule for the prevention or treatment of metabolic syndrome, and further study is necessary to clarify the mechanism how TRB3 induces insulin resistance by regulating insulin signal transduction via adipose tissue, and finally leading to metabolic syndrome.

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References


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