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SHORT COMMUNICATION

Aberrant hepatic *TRIB3* gene expression in insulin-resistant obese humans

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Abstract
Aims/hypothesis The pseudokinase tribbles homologue 3 (Drosophila) (TRIB3) negatively interferes with insulin-mediated phosphorylation and activation of v-akt murine thymoma viral oncogene homologue 1 (AKT1, also known as protein kinase B). Animal studies have shown that *Trib3* expression was higher in the fasting state and in animal models of diabetes, promoting hyperglycaemia presumably by increasing glucose production in the liver. Less is known about the role of TRIB3 in insulin resistance in humans, although a gain-of-function mutation associated with abnormalities related to insulin resistance has been described in *TRIB3*.
Methods We determined hepatic mRNA expression of *TRIB3* and selected genes encoding enzymes, transcription factors and coactivators involved in glucose homeostasis. We also determined biochemical variables of intermediary metabolism in obese patients with varying degrees of insulin resistance.
Results In our study population hepatic *TRIB3* mRNA expression was associated with surrogate markers of insulin resistance. *TRIB3* expression was significantly increased in a subgroup with high HOMA of insulin resistance (HOMA-IR) compared with a low HOMA-IR group (p=0.0033). *TRIB3* transcript levels were correlated with *PEPCK* (also known as *PCK2*) mRNA expression (p=0.0014) and mRNA expression of *PPARGC1A* (p=0.0020), *PPARGC1B* (p<0.0001), *USF1* (p=0.0017), *FOXO1* (p=0.0003) and *SREBP-1c* (also known as *SREBF1*; p=0.0360). Furthermore ligands of peroxisome proliferator-activated receptor α/retinoid X receptor and overexpression of its coactivator *PPARGC1A* as well as overexpression of *SREBP-1c* and its coactivator *PPARGC1B* increased *TRIB3* promoter activity in HepG2 cells.
Conclusions/interpretation We have found evidence for a role of aberrant hepatic *TRIB3* transcript levels in insulin resistance in obese humans and identified potential transcriptional pathways involved in regulation of *TRIB3* gene expression in the liver.

Keywords Gene expression · Insulin resistance · Transcription · Tribbles 3

Abbreviations

AKT1	V-akt murine thymoma viral oncogene homologue 1
CRP	C-reactive protein
HOMA-IR	HOMA of insulin resistance
PPARA	Peroxisome proliferator-activated receptor α
PPARGC1A	Peroxisome proliferator-activated receptor γ coactivator 1 alpha
PPARGC1B	Peroxisome proliferator-activated receptor γ coactivator 1 beta
SREBP-1c	Sterol regulatory element binding protein 1c
TRIB3	Tribbles homologue 3

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Introduction

Tribbles homologue 3 (TRIB3), the mammalian homologue of Drosophila tribbles, is classified as a pseudokinase with a truncated kinase domain that lacks ATP-binding and catalytic residues. An inhibitory role for TRIB3 in insulin signal transduction has been suggested, as it was shown to bind to the pleckstrin homology domain of the insulin-responsive Ser-Thr kinase, v-akt murine thymoma viral oncogene homologue 1 (AKT1), thereby preventing its membrane association, Thr^{308} phosphorylation and activation via upstream kinases [1, 2]. Although, in mice, deletion of *Trib3* did not produce major disturbances in insulin signalling and glucose homeostasis [3], and in cultured rat hepatocytes adenoviral *Trib3* overexpression failed to affect insulin mediated AKT1 phosphorylation [4], several other studies have reported data consistent with a role for *Trib3* in insulin resistance. *Trib3* expression was increased in livers of *db/db* mice, a rodent model of type 2 diabetes [1]. Furthermore hepatic *Trib3* gene knock-down enhanced insulin sensitivity, whereas adenovirus-mediated hepatic overexpression of *Trib3* promoted hyperglycaemia and glucose intolerance in wild-type mice [5]. In the fasted state, TRIB3 was increased, thereby preventing residual insulin signalling and promoting glucose output by the liver [1]. Furthermore, increased expression of *Trib3* has been implicated in ethanol-induced hepatic insulin resistance [2]. Most recently, a protein termed adaptor protein, phosphotyrosine interaction, PH domain and leucin zipper containing 1, which mediates adiponectin signalling, was shown to enhance insulin-stimulated activation of AKT1 and suppression of gluconeogenesis by blocking the interaction of AKT1 with TRIB3 through direct competition [6].

In humans, associations of a gain-of-function *TRIB3* Glu84Arg missense polymorphism with phenotypes related to insulin resistance have been described [7]. Furthermore, overexpression of the variant Arg84 allele enhanced inhibition of insulin-mediated AKT1 phosphorylation in HepG2 cells [7] and was associated with impaired insulin signalling and nitric oxide production in human endothelial cells [8]. Because of a possible role of TRIB3 in insulin resistance in humans, we measured hepatic mRNA expression of *TRIB3* in obese patients with varying degrees of insulin resistance and determined associations with biochemical variables and hepatic transcript levels of genes involved in transcriptional regulation and glucose homeostasis.

Methods

The study included 93 obese patients undergoing weight-reducing surgery. Of these, 54 underwent a gastric banding procedure and 39 a gastric bypass procedure, in both cases under general anaesthesia (propofol and remifentanil). Participants were included if they had fasting blood glucose levels <7.0 mmol/l, no history of diabetes or use of glucose- or lipid-lowering medication, and no weight changes >3% during the previous 2 months. All study participants provided informed consent and study protocols were approved by the local Ethics Committee. Adipose tissue mass, diameter of abdominal subcutaneous adipose tissue and visceral adipose tissue, and grading of fatty liver disease were determined as described [9]. Tissue biopsies were obtained from liver as well as visceral and subcutaneous adipose tissue at ~20 min after induction of anaesthesia in patients undergoing gastric banding procedure and at ~60 min in those undergoing gastric bypass, respectively; biopsies were collected in RNA-later (Ambion, Austin, TX, USA).

Plasma glucose, insulin, cholesterol, triacylglycerol, HDL-cholesterol and C-reactive protein (CRP) were determined as described [10]. Plasma adiponectin was measured using an immunoassay (Human Adiponectin/Acrp30; R&D Systems, Wiesbaden-Nordenstadt, Germany). HOMA-IR was calculated as described previously [9]. Total RNA was isolated and reverse-transcribed as described [10]. Transcript levels of genes listed in Electronic supplementary material (ESM) Table 1 were quantified in duplicate using TaqMan gene expression assays (Applied Biosystems, Warrington, UK) and a real-time PCR detector (iCycler iQ Multicolour; Bio-Rad, Hercules, CA, USA). Constitutively expressed acidic ribosomal protein p0 mRNA was measured for normalisation of mRNA abundance.

The TRIB3-Prom-Luc plasmid was generated using 5′-GAGACG CGTTGCAGGGATACAAAGCTCGTGA-3′ (−3073 to −3051) and 5′-AGACTCGAGAGT ACCTCG CCCCGTCGTTCC-3′ (489 to 510) as forward and reverse primers, respectively, to amplify a 3,583 bp DNA fragment that was cloned into the pGL3-Basic Vector (Promega, Madison, WI, USA) using restriction endonucleases MluI and XhoI. Numbers in parentheses refer to primer positions relative to the transcriptional start site (GenBank accession number NM_021158). The *PPARGC1A* (also known as *PGC-1α*), *PPARGC1B* (also known as *PGC-1β*) and nuclear active *SREBP-1c* expression constructs have been described previously [10].

Human HepG2 hepatoma cells were grown and transfected as described [10]. Unless otherwise indicated, we used 1 μg of reporter plasmid, 0.5 μg of expression plasmids and 1 ng of pRL-TK plasmid (Promega) as transfection control. Drugs at concentrations of 10 μmol/l for retinoic acid and WY 14.643 were added immediately before transfection. Cells were collected 24 h after transfection, and firefly and *Renilla* luciferase activities were measured as described [10].

Table 1 Associations of hepatic *TRIB3* mRNA with transcript levels of selected transcription factors, coactivators and genes involved in hepatic gluconeogenesis

Transcript	Regression coefficient (SE)	*p* value
PPARGC1A	0.309 (0.097)	0.0020[a]
PPARGC1B	0.451 (0.091)	0.000003[a]
SREBP-1c	0.225 (0.105)	0.0360
USF1	0.316 (0.097)	0.0017[a]
FOXO1	0.362 (0.096)	0.0003[a]
PEPCK	0.323 (0.098)	0.0014[a]

Values are adjusted for age, sex and HOMA-IR, $n>85$ participants

[a] Significant after the Bonferroni correction

Differences in continuous variables between HOMA of insulin resistance (HOMA-IR) groups were ascertained by analysis of variance. To meet the equal variance and normality assumptions of analysis of variance, logarithmic transformations for plasma triacylglycerol, CRP, and hepatic and adipose tissue transcript levels were done. We adjusted measurements, by multiple regression for concomitant effects of sex and age. Categorical data were summarised by frequencies and analysed by the χ^2 test. To estimate associations between hepatic *TRIB3* mRNA levels and other hepatic transcripts levels or biochemical variables, we used multivariate linear regression. Logarithmic transformation of transcript variables was used to fulfil general linear model assumptions. Reported *p* values are two-tailed. To test the significance of multiple comparisons, the Bonferroni correction was used.

Results and discussion

A detailed description of clinical and biochemical variables of our obese study population, characterised by varying degrees of insulin resistance, is shown in ESM Table 2. Among variables commonly associated with insulin resistance, sex and age-adjusted hepatic *TRIB3* mRNA levels showed associations with plasma insulin ($p=0.0031$), HOMA-IR ($p=0.0029$), plasma triacylglycerol ($p=0.0089$) and hepatic steatosis score ($p=0.0087$). An inverse association was observed with plasma adiponectin ($p=0.0345$; ESM Table 3). However, the associations with steatosis score, adiponectin or plasma triacylglycerol were not maintained after adjustment for HOMA-IR. No associations were noted with plasma glucose, HDL-cholesterol and CRP or obesity indices such as BMI and fat mass (data not shown).

To elucidate the role of *TRIB3* in insulin resistance in humans, we studied possible associations of hepatic, visceral and subcutaneous tissue transcript levels of *TRIB3* in subgroups of 32 obese participants with HOMA-IR<2.0 and of 32 obese participants with HOMA-IR >5.0. The number of participants undergoing gastric banding or gastric bypass surgery was similar in the high- and low-HOMA-IR groups (ESM Table 4). Participants included in the high HOMA-IR group had significantly higher values for triacylglycerol and lower values for HDL-cholesterol, adiponectin and both visceral and subcutaneous *SLC2A4* mRNA expression (ESM Table 4). Hepatic AKT1 phosphorylation, as determined by western blot analysis, was significantly reduced in participants with a high HOMA-IR ($p<0.05$; ESM Fig. 1). The sex- and age-adjusted comparison showed higher hepatic *TRIB3* mRNA levels in the high HOMA-IR group than in the low HOMA-IR group ($p=0.0033$; ESM Table 4). This difference remained significant

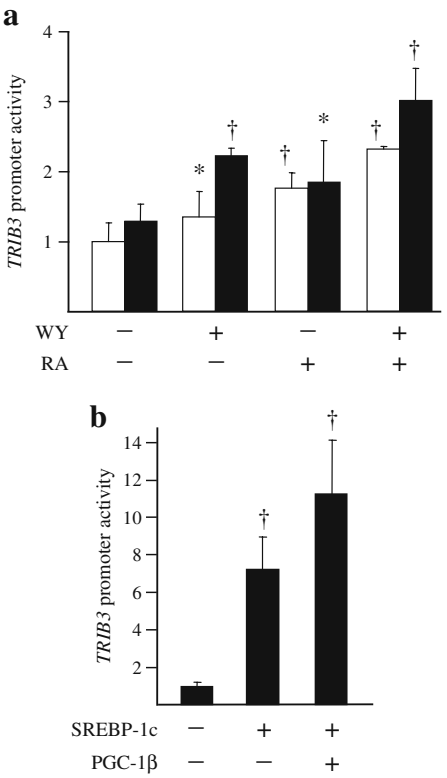

Fig. 1 PPARα/retinoic X receptor, SREBP-1c and their coactivators PPARGC1A and PPARGC1B respectively, are involved in the transcriptional regulation of human *TRIB3* gene expression in HepG2 cells. **a** HepG2 cells were transiently transfected with the TRIB3-Prom-Luc reporter plasmid or co-transfected with a human *PPARGC1A* (also known as *PGC-1α*) expression construct in the absence or presence of WY14.643 (WY) and retinoic acid (RA; 10 nmol/l each) as indicated. *$p<0.05$ and †$p<0.005$ for basal (−) compared with drug-stimulated (+) reporter gene activities in the absence (white bars) or presence (black bars) of *PPARGC1A* overexpression. **b** HepG2 cells were transfected with TRIB3-Prom-Luc or co-transfected with *SREBP-1c* and/or *PPARGC1B* (also known as *PGC-1β*) expression constructs as indicated. Basal TRIB3-Prom-Luc reporter gene activity in the absence of expression plasmids or ligands was set to 1.0. Data are presented as fold change ± SD; *$p<0.05$ and †$p<0.005$

after adjustment for the surgical procedure (p=0.0053; ESM Table 4). In addition, increased *TRIB3* transcript abundance was also observed in visceral tissue samples (p=0.0029). A similar trend, albeit not significant, was obtained in subcutaneous tissue specimens.

Sex-specific analysis in the larger group of women also showed higher *TRIB3* mRNA levels in the high HOMA-IR group (2.62±2.23) than in the low HOMA-IR group (1.15±1.05; p=0.0195). A similar trend was observed in the smaller group of men (p=0.1612). No significant interaction between sex and *TRIB3* mRNA was observed. Hepatic *TRIB3* expression displayed a strong correlation with *PEPCK* (also known as *PCK2*) mRNA expression (Table 1), which is consistent with previous observations demonstrating that adenoviral mediated overexpression of *TRIB3* resulted in increased glucose output from the liver [1]. Our data obtained in humans are therefore consistent with a role for *TRIB3* in insulin resistance in obese humans.

To gain insight into the transcriptional networks involved in the regulation of *TRIB3* expression in humans, we measured hepatic mRNA levels of selected transcription factors and coactivators. In our study population, *TRIB3* transcript levels, adjusted for age, sex and HOMA-IR, correlated with *PPARGC1A*, *PPARGC1B*, *USF1* and *FOXO1* mRNA expression levels, as well as, unexpectedly, with transcripts encoding sterol regulatory element binding protein 1c (*SREBP-1c*; Table 1). No associations were observed with *PPARA*, *LXRA* (also known as *NR1H3*), *USF2*, *CHREBP* (also known as *MLXIPL*) and *CEBPA* transcripts (data not shown). Previous studies in mice showed that *Trib3* transcription is increased via co-activation of peroxisome proliferator-activated receptor α (PPARA) by peroxisome proliferator-activated receptor γ coactivator 1 alpha (PPARGC1A, also referred to as PGC-1α) [5]. To determine whether transcription from the human *TRIB3* promoter is activated by a similar mechanism, we transiently transfected HepG2 cells with a luciferase reporter construct driven by the human *TRIB3* promoter (TRIB3-Prom-Luc). Incubation of transfected cells with PPARA and/or retinoic X receptor ligands (WY 14.643 and 9-*cis* retinoic acid, respectively) increased reporter gene activity, which was further augmented upon co-transfection with a *PPARGC1A* expression plasmid (Fig. 1a). Hence, PPARGC1A coactivates the PPARA-mediated transcription of the human *TRIB3* gene.

A strong association was observed between *TRIB3* mRNA and transcript levels of the transcriptional co-activator peroxisome proliferator-activated receptor γ co-activator 1 beta (PPARGC1B, also referred to as PGC-1β, which, in contrast to PPARGC1A, has been shown to bind and directly co-activate SREBPs [11]. Despite a modest association between *TRIB3* transcripts and transcripts encoding SREBP-1c, recent studies have shown that nuclear import of SREBP-1c is enhanced by endoplasmic reticulum stress, which is implicated in insulin resistance [12]. We therefore directly determined effects of SREBP-1c and PPARGC1B on transcriptional regulation of the human *TRIB3* gene, in co-transfection experiments using HepG2 cells. The TRIB3-Prom-Luc construct was transiently transfected together with a nuclear active *SREBP-1c* expression plasmid. Nuclear active SREBP-1c increased the basal transcriptional activity and coexpression of *PPARGC1B* further enhanced the SREBP-1c-mediated activation of the TRIB3-Prom-Luc reporter construct (Fig. 1b), indicating a role for SREBP-1c and PPARGC1B in the regulation of human *TRIB3* gene transcription.

Components of the insulin-signalling pathway have been extensively studied over the past years in an attempt to identify their physiological contributions to the regulation of glucose homeostasis. TRIB3 is an inhibitor of the insulin-responsive AKT1 kinase and has been implicated in insulin resistance in several studies using cell lines and animal models [1, 2, 5, 6]. In the present study, we provide initial evidence for an aberrant hepatic expression of *TRIB3* in insulin-resistant human study participants. These results should, however, be interpreted with caution, as this phenomenon might be restricted to the obese population. Correlations of *TRIB3* transcript levels with mRNA levels of distinct transcription factors and coactivators, together with our observations from cell culture, may help to unravel the underlying complex transcriptional pathways that result in human insulin resistance.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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