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BASIC SCIENCE RESEARCH

SIRT1 Transcription Is Decreased in Visceral Adipose Tissue of Morbidly Obese Patients with Severe Hepatic Steatosis

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Abstract
Background Visceral adipose tissue is known to release greater amounts of adipokines and free fatty acids into the portal vein, being one of the most predictive factors of nonalcoholic fatty liver disease (NAFLD). Our study has the purpose to evaluate sirtuin 1 (SIRT1), adiponectin, Forkhead/winged helix (FOXO1), peroxisome proliferator-activated receptor (PPAR)γ1–3, and PPARβ/δ mRNA expression in morbidly obese patients in three different lipid depots: visceral (VAT), subcutaneous (SAT), and retroperitoneal (RAT). Recent studies suggest that SIRT1, a NAD^+-dependent deacetylase, protects rats from NAFLD.
Methods We divided the patients in two groups: those with slight or moderate steatosis (hepatic steatosis, HS) and other comprising individuals with severe steatosis associated or not with necroinflammation and fibrosis (severe hepatic steatosis, SHS). The adipose tissue depots were obtained during bariatric surgery. Total RNAs were extracted using TRIzol. The amount of genes of interest was determined by quantitative real-time polymerase chain reaction.
Results When comparing the two groups of patients, a decrease in SIRT1 was observed in VAT of morbidly obese patients in SHS group ($p=0.006$). The mRNA expression of the other genes showed no differences in VAT. No difference was found either in SAT or in RAT for all genes in the study. In addition, the homeostasis model assessment for insulin resistance (HOMA-IR) value was higher in SHS group compared to HS ($p=0.006$). Also, our results show that the mRNA expression of SIRT1 and the value of HOMA-IR were positively correlated in VAT of SHS patients ($r=0.654$; $p=0.048$).
Conclusions Downregulation of SIRT1 mRNA expression in VAT of SHS could be possible impairing mitochondria biogenesis and fatty acid oxidation, promoting severe steatosis in obese patients. Our results provide a possible proof of SIRT1 protective potential in VAT against NAFLD in humans.

Keywords Adipose tissue depots · SIRT1 · Hepatic steatosis · Visceral adipose tissue

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Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
AT	Adipose tissue
FFA	Free fatty acid
RAT	Retroperitoneal adipose tissue
SAT	Subcutaneous adipose tissue
VAT	Visceral adipose tissue
FOXO1	Forkhead/winged helix
SIRT1	Sirtuin 1
PPAR	Peroxisome proliferator-activated receptors
qRT-PCR	Quantitative real-time polymerase chain reaction

Introduction

Obesity is a condition described as excess of body weight, more specifically of adipose tissue (AT) [1, 2]. Evidence suggests that there are differences in biochemical and molecular characteristics of different lipid depots [3–6]. Visceral AT has greater lipolytic potential than subcutaneous AT, and the release of free fatty acid (FFA) from visceral fat depots directly into the portal circulation is one of the mechanisms of hepatic injury [7]. Busetto et al. found a strong association between visceral fat accumulation and liver steatosis in morbidly obese women [8].

Several studies in selected cohorts have extensively demonstrated that obesity may play a role in the development and progression of steatosis [9]. Nonalcoholic fatty liver disease (NAFLD) is characterized as liver fat accumulation [10]. Liver fat deposition may either be isolated (steatosis) or associated with a variable degree of necroinflammation and fibrosis (nonalcoholic steatohepatitis, NASH) [7, 9].

Sirtuins (SIRT) are a mammalian NAD^+-dependent histone deacetylase family [11]. There are seven mammalian sirtuins, SIRT1–7, of which SIRT1 has been the most studied [12, 13]. SIRT1 has been implicated in the control of lipid and glucose metabolism [14–16]. A recent study suggests that moderated overexpression of SIRT1 protects mice from NAFLD [17]. Another study found that the expression of SIRT1 in liver is significantly reduced in NAFLD induced by high-fat diet in rats [18]. In addition, the protective role of SIRT1 in oxidative stress, which is involved in the development of NASH [9], has been recently evaluated [19–22].

Adiponectin is an adipocyte-derived protein that has anti-obesity, antidiabetic, and anti-inflammatory properties and is considered to have a hepatoprotective function [23]. In human patients with NAFLD, serum adiponectin levels were found to be decreased [24]. The transcriptional factor Forkhead/winged helix (FOXO1) had been recently associated with NAFLD [25–27]. Valenti et al., studying 84 patients with liver fat accumulation, found that FOXO1 hepatic expression was increased in patients with steatohepatitis [28].

The nuclear peroxisome proliferator-activated receptor (PPAR) family has been intensively studied in the past several years. The PPAR family comprises three isotypes: PPARα, PPARγ, and PPARβ/δ [29]. PPARγ is highly expressed in adipose tissue and plays a key role in adipogenic activity [30, 31]. It has been reported that SIRT1 represses PPARγ in white AT by docking with its cofactors nuclear receptor corepressor [14, 32]. PPARγ mRNA levels are usually elevated in steatotic mice liver [33]. Little is known about PPARβ/δ related to NAFLD. A recent study shows that treatment with PPARβ/δ agonist or overexpression of this nuclear receptor markedly reduced intracellular lipid accumulation [34].

Driven by the need for potent and safe options to treat obesity and its related abnormalities as steatosis, numerous efforts are currently under way to achieve a better understanding of molecular networks controlling cellular glucose, lipid, and energy metabolism [17]. The sequence of events and the mediators linking AT, especially visceral fat, to liver disease progression are not well defined [7]. Different abdominal (visceral, subcutaneous, and retroperitoneal) AT depots have different metabolisms and play a yet unknown role in obesity and NAFLD. In this study, we investigated the relationship between SIRT1, adiponectin, FOXO1, PPARγ1-3, and PPARβ/δ relative mRNA expression in various ATs of morbidly obese patients with different degrees of hepatic steatosis.

Materials and Methods

Samples

Samples of visceral AT (VAT), subcutaneous AT (SAT), and retroperitoneal AT (RAT) were obtained from 24 morbidly obese patients (body mass index-BMI≥40 kg/m^2) who underwent open Roux-en-Y gastric bypass at the Center of Morbid Obesity of the São Lucas Hospital of the Pontifícia Universidade Católica do Rio Grande do Sul (Brazil). The incision was made vertically along the midline, above umbilicus, with a scalpel blade. At this region, SAT sample was collected. To access the cavity, the surgeons opened the aponeurosis with an electrocautery, and preperitoneal AT resection was made, which was called retroperitoneal fat. Next, VAT collection was made in omentum, without cauterization. Subjects with a history of excessive drinking or other specific liver diseases were excluded from the study. Patients' weight had been stable for at least 1 year. No subjects were taking any medications affecting adipocyte metabolism.

Patients were divided in two groups according to fat liver accumulation levels, diagnosed by liver biopsy material collected during bariatric surgery [9, 35]. Steatosis degree was classified according to Burt et al. [36]. The hepatic steatosis (HS) group was formed by patients with slight to moderate steatosis (n=12), and the severe hepatic steatosis (SHS) group was formed by patients with severe steatosis associated or not with necroinflammation and fibrosis (n=12). The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul (No. 2007/936). All subjects were informed about the aim of the study and signed the informed consent form.

Analysis of mRNA Expression

AT samples were collected and immediately immersed in TRIzol reagent for total RNA extraction. Approximately

2 μg of total RNA were added to each cDNA synthesis reaction using 200 U of the M-MLV Reverse Transcriptase (Invitrogen) in a final volume of 25 μL. Reactions were performed at 42°C for 1 h using the primer T23V (5′-TTT TTT TTT TTT TTT TTT TTT TTV-3′). Quantitative real-time polymerase chain reaction (qRT-PCR) amplification was carried out using specific primer pairs designed with the Primer 3 calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) and synthesized by Prodimol (São Paulo, Brazil). The sequences of the primers used are listed in Table 1. An Applied-Biosystem 7500 real-time cycler was used to carry out the qRT-PCRs. Reaction settings were formed by an initial denaturation step of 5 min at 95°C followed by 40 cycles of 15 s at 95°C, 10 s at 60°C, 15 s at 72°C, and 35 s at 60°C. Samples were held for 15 s at 95°C for annealing and then heated for 1 min at 60°C with a ramp of 0.1°C/s to acquire data to produce the denaturing curve of the amplified products. The qRT-PCRs were made in a final volume of 20 μL, formed of 10 μL of each reverse transcription sample diluted 40 to 100 times, 2 μL of PCR buffer diluted ten times, 1.2 μL of 50 mM $MgCl_2$, 0.1 μL of 5 mM deoxyribonucleotide triphosphate, 0.4 μL of 10 μM primer pairs, 4.25 μL of water, 2.0 μL of SYBR green (1:10,000; Molecular Probe), and 0.05 μL of Platinum Taq DNA polymerase (5 U/μL; Invitrogen).

Data Analyses

We quantified gene expression using the $2^{-\Delta\Delta Ct}$ (threshold cycle) method [37]. For each sample analyzed in quadruplicate, a ΔC_T value was obtained by subtracting the beta-2-microglobulin (B2M) C_T value from the C_T value of the gene of interest. The ΔC_T mean value obtained for the HS group was used to calculate the ΔΔCt of each gene and tissue ($2^{-\Delta\Delta Ct}$).

The extensive clinical and laboratory data routinely collected for each patient are shown in Table 2. The homeostasis model assessment for insulin resistance (HOMA-IR) is a mathematical model that measures values of insulin resistance in humans. It is calculated as follows: HOMA-IR = fasting plasma insulin level (microunits per milliliter) × (fasting plasma glucose level (milligrams per deciliter) × 0.05551)/22.5 [38].

Statistics

To compare SIRT1, adiponectin, FOXO1, and PPARs mRNA expression between HS and SHS patients, we used the nonparametric Mann–Whitney *U* test. Data are shown as median ± confidence interval. To compare anthropometric and biochemical parameters of morbidly obese patients between the HS and SHS groups, we used the parametric independent sample *T* test. Data are shown as mean ± standard deviation. The correlations were examined by the nonparametric Spearman's rank test. Differences were considered statistically significant at $p \leq 0.05$. All data were calculated using the SPSS program (version 15.0).

Results

When evaluating anthropometric data (BMI, waist, and hip), no significant difference was observed between HS and SHS patients (Table 2). Considering age, HS patients were a little younger than SHS patients were, but this difference is not statistically significant. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values showed no difference between the two groups, but both mean values were above normal reference values in the SHS group [39]. The serum triglyceride mean value of HS patients was smaller than in SHS, but not statistically different. Values of total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were not different between both groups. However, HOMA-IR values were substantially different in HS when compared with SHS ($p = 0.006$). The

Table 1 Oligonucleotides used in qRT-PCR reactions

B2M	Forward 5′-TGCTGTCTCCATGTTTGATGTATCT-3′
	Reverse 5′-TCTCTGCTCCCCACCTCTAAGT-3′
SIRT1	Forward 5′-GAGTGGCAAAGGAGCAGA-3′
	Reverse 5′-TCTGGCATGTCCCACTATC-3′
Adiponectin	Forward 5′-TGGTGAGAAGGGTGAGAA-3′
	Reverse 5′-AGATCTTGGTAAAGCGAATG-3′
FOXO1	Forward 5′-TGGACATGCTCAGCAGACATC-3′
	Reverse 5′-TTGGGTCAGGCGGTTCA-3′
PPARγ1–3	Forward 5′-AGGCCATTTTCTCAAAC-3′
	Reverse 5′-AGAAATGCTGGAGAAGTCAACA-3′
PPARβ/δ	Forward 5′-AATGCCTACCTGAAAAACTTCAAC-3′
	Reverse 5′-GTGCACGCTGATTCCTTGT-3′

Table 2 Anthropometric and biologic parameters of HS and SHS obese patients

	HS group mean (±SE)	SHS group mean (±SE)
BMI (kg/m^2)	46.76 (±2.96)	47.57 (±2.68)
Waist (cm)	130.00 (±6.26)	137.08 (±5.38)
Hip (cm)	141.50 (±5.31)	141.08 (±7.07)
Age (years)	34.83 (±3.21)	36.08 (±3.32)
ALT (U/L)	28.91 (±5.09)	39.67 (±10.55)
AST (U/L)	41.73 (±8.48)	59.00 (±14.24)
Triglycerides (mg/dL)	147.8 (±26.43)	159.75 (±21.17)
Total cholesterol (mg/dL)	189.0 (±9.06)	185.08 (±12.20)
HDL-C (mg/dL)	46.80 (±1.88)	49.09 (±5.14)
LDL-C (mg/dL)	129.6 (±8.04)	111.36 (±11.91)
HOMA-IR (mmol/L×μU/mL)	5.01 (±0.74)	7.02 (±1.58)*

To compare anthropometric and biologic data between obese patients with HS and the SHS groups, the parametric independent sample *T* test was used to calculated *p* values

*$p \leq 0.05$ (represents a statistically significant difference between HS and SHS)

mean value of HOMA-IR was 5.01±0.74 in HS and 7.02± 1.58 in SHS.

The expression of SIRT1, adiponectin, FOXO1, PPARγ1-3, and PPARβ/δ mRNA in VAT, SAT, and RAT of morbidly obese subjects was assessed by qRT-PCR. Table 3 reflects the relative SIRT1 mRNA expression profile of morbidly obese patients in the three ATs. In VAT, SIRT1 mRNA expression was statistically different between the HS and SHS groups ($p=0.006$). In this tissue, SIRT1 amounts were lower in SHS (0.12) when compared with HS (1.04). SIRT1 relative mRNA expression in SAT and RAT did not maintain the statistically difference between the two groups of patients (Table 3).

Table 3 also presents adiponectin and FOXO1 relative mRNA expression of both groups of patients. The expression of adiponectin and FOXO1 in SAT and VAT was not

Table 3 Comparative analyses of SIRT1, adiponectin, FOXO1, PPARγ1–3, and PPARβ/δ mRNA levels between HS and SHS obese patients in VAT, SAT, and RAT

Gene	VAT (median±confidence interval)	SAT (median±confidence interval)	RAT (median±confidence interval)
SIRT1			
Group HS	1.04 (0.37–3.49)	1.39 (0.19–4.01)	0.82 (−0.58–4.60)
Group SHS	0.12 (0.09–0.57)*	0.80 (0.03–2.62)	0.60 (0.28–1.11)
Adiponectin			
Group HS	1.44 (0.50–2.14)	1.97 (−1.46–8.24)	ND
Group SHS	1.77 (0.23–3.57)	5.36 (−1.97–11.84)	ND
FOXO1			
Group HS	1.37 (0.36–2.64)	1.04 (0.56–1.65)	ND
Group SHS	1.84 (1.27–2.47)	0.59 (0.28–0.81)	ND
PPARγ1–3			
Group HS	1.38 (−0.66–5.93)	1.14 (0.27–3.10)	1.41 (0.26–2.87)
Group SHS	0.75 (0.20–1.75)	0.65 (0.01–1.89)	0.64 (0.44–0.90)
PPARβ/δ			
Group HS	1.43 (0.28–2.75)	1.35 (0.01–2.92)	1.30 (0.02–3.43)
Group SHS	0.80 (0.20–1.27)	1.36 (−0.20–4.86)	0.76 (0.17–1.17)

To compare the expression pattern of SIRT1, adiponectin, FOXO1, PPARγ1–3, and PPARβ/δ mRNA in different abdominal AT—VAT, SAT, and RAT—in the HS and SHS groups, the nonparametric Mann–Whitney *U* test was used. Data are shown as median±confidence interval. Relative expressions were normalized against B2M (ΔC_T) and calibrated to the mean value of HS of each gene and tissue ($2^{-\Delta\Delta Ct}$)

ND not determined

*$p \leq 0.05$ (a statistically significant difference between HS and SHS)

statistically different between the two groups. We did not analyze adiponectin and FOXO1 mRNA expression in RAT.

Furthermore, we analyzed PPARγ1-3 and PPARβ/δ expression in the three AT depots (Table 3). When comparing the PPARγ1-3 and PPARβ/δ relative mRNA expression between HS and SHS, we found that there was no statistically significant difference in VAT, SAT, and RAT between these two groups.

Gene expression in VAT, anthropometric, and biochemical parameters of morbidly obese patients were correlated. We found a positive correlation between SIRT1 mRNA expression and HOMA-IR value (r=0.654, p=0.048) in SHS group.

Discussion

Adipose tissue is not simply a storage depot for energy but rather an endocrine organ. VAT is known to release large quantities of adipokines and FFAs into the portal vein, being one of the most predictive factors of NAFLD [7, 9]. The sequence of events and the mediators linking abdominal fat to liver disease progression are not well defined [7]. Information on site-related gene expression of SIRT1, adiponectin, FOXO1, and PPARs in human AT is limited. The aim of this study was to compare the relative mRNA expression of SIRT1, adiponectin, FOXO1, PPARγ1–3, and PPARβ/δ in morbidly obese patients with different grades of hepatic steatosis. We evaluated gene expression in various abdominal tissues—visceral, subcutaneous, and retroperitoneal—to understand the role of different adipose depots in NAFLD obese patients.

Almost nothing is known about PPARs expression in human adipose tissue related to NAFLD. Transgenic expression of an activated form of PPARβ/δ in AT produces lean mice that are resistant to obesity and tissue steatosis [40]. We found no difference in PPARγ1–3 and PPARβ/δ mRNA expression in various abdominal adipose depots between HS and SHS morbidly obese patients. A recent study published by our group suggests a probable imbalance between PPARβ/δ (involved in fatty acid oxidation) and PPARγ1–3 (related to adipogenesis) expression regulating adipocytes development in obesity [41]. PPAR expression in AT could be associated with obesity [41] but, according to our present results, may be not related to NAFLD severity.

An increased number of evidence has linked NAFLD and metabolic syndrome with an excess of visceral fat [7, 9]. Although the data are mainly epidemiologic, the pathogenesis of NAFLD and metabolic syndrome seems to have a common pathophysiologic mechanism [19]. VAT tissue releases greater quantities of adipokines and excess FFAs into the portal vein, exposing the liver to higher FFA concentrations [7, 9]. There is one study that analyzed adiponectin expression in human AT related to NAFLD [42]. By the other side, neither study has been yet described about SIRT1 and FOXO1 mRNA expression in human AT of morbidly obese patients related to NAFLD. Our results indicate a reduced SIRT1 mRNA expression in SHS compared to HS morbidly obese patients, only in VAT. We did not find any difference in adiponectin and FOXO1 mRNA expression between HS and SHS in this tissue.

A few number of studies have suggested the protective effects of SIRT1 on the pathogenesis of NAFLD. Pfluger et al. found that transgenic mice moderately overexpressing SIRT1, when on a high fatty acid diet, are almost entirely protected from hepatic steatosis [17]. Yamazaki et al., using a SIRT1 specific activator (SRT1720), found that SRT1720 treatment significantly reduced triglycerides and aminotransferase levels, lipogenic genes expression (acetyl-CoA carboxylase and fatty acid synthase), and FFA serum levels in MSG mice [43].

Also, SIRT1 is involved in fatty acid mobilization in AT [32] and has been related to the stimulus of mitochondrial biogenesis [12]. Recent studies emphasize the role of mitochondrial dysfunction in the development and progression of NAFLD [17, 44]. Maassen et al. have recently published the hypothesis that one of the functions of mitochondria in AT is to prevent leakage of fatty acids into the circulation, which could attenuate the development of ectopic triacylglycerol deposits in the liver [45]. Therefore, downregulation of SIRT1 in VAT of obese patients with severe steatosis could impair mitochondrial activity, increase the release of FFA into the portal circulation, and augment the hepatic oxidative stress and inflammation-related genes expression which contribute to the development of steatohepatitis [17, 44, 46].

The present study is the first report to analyze SIRT1, adiponectin, FOXO1, PPARγ1–3, and PPARβ/δ expression in SAT of morbidly obese patients with different degrees of steatosis. A previous study had discussed the role of subcutaneous fat in NAFLD [17], but we found no difference in gene expression in SAT between simple and severe steatosis. There are no studies on RAT related to NAFLD. No statistically significant difference was observed in SIRT1, adiponectin, FOXO1, PPARγ1–3, and PPARβ/δ mRNA expression in this tissue in HS and SHS patients.

In the present study, we evaluated the gene mRNA expression levels, which may not necessarily equate to protein synthesize. Therefore, further studies are necessary to reveal this.

The molecular mechanisms underlying the relationship between fat liver accumulation and insulin resistance have not been evaluated in humans [28]. NAFLD was significantly associated with HOMA-IR, which is more marked in the presence of NASH [28]. Park et al., studying the

prevalence and risk factors of nonalcoholic fatty liver disease among Korean adults, found that NAFLD was significantly associated with elevated HOMA-IR [47]. In our study, we observed that the HOMA-IR mean value in HS was statistically smaller in SHS and that in SHS group, SIRT1 correlated positively with HOMA-IR, suggesting that lower SIRT1 mRNA expression could also be involved in glucose and insulin metabolism damaging in SHS patients.

In summary, the present study shows that SIRT1 relative mRNA expression was lower in VAT of SHS patients when compared with HS patients. We suggest that downregulation of SIRT1 in VAT of SHS could impair mitochondria biogenesis and fatty acid oxidation, promoting severe hepatic steatosis in morbidly obese patients. VAT plays an important role in the pathogenesis of hepatic steatosis because it supplies fat to the liver via the portal vein, and SIRT1 could prevent FFAs from being released from VAT. Nevertheless, our study really provided a molecular evidence for the use of SIRT1 as a therapy for the management of NAFLD. More research is necessary to better the association between VAT and steatosis.

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