Vaspin Gene Expression in Rat Adipose Tissue: 
Relation to Obesity and Exercise

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Abstract

Visceral adipose fat has been claimed by many studies to be the link between obesity and insulin resistance through the released adipokines. The aim of this study was to assess the effect of obesity and exercise on expression of vaspin as one of the recent adipokines in abdominal subcutaneous and visceral fat of diet-induced obese (DIO) rats and DIO rats performing 3 weeks swimming exercise (DIO+EXE) compared to control and control+exercise (C+EXE) groups and to evaluate vaspin’s correlation with the visceral fat weight, insulin resistance and serum leptin level.

Vaspin mRNA expression was detected in the obese and not in the control group. Moreover it was frequently detected in the DIO abdominal visceral fat compared to the subcutaneous one positively correlated with the body weight and insulin resistance, suggesting a role of vaspin in obesity induced insulin resistant states.

Vaspin expression was absent in C+EXE group. Also visceral vaspin mRNA was unchanged in DIO+EXE rats (26.7% versus 40% of cases in DIO), in spite the significant improvements of insulin resistance parameters and serum leptin compared to DIO group denoting that vaspin expression is not related to exercise but rather to the increase in body weight which was not significantly changed by exercise alone.

In Conclusion: Visceral vaspin expression increases in obesity associated with insulin resistance and is unchanged by exercise. Further investigations into the molecular links between vaspin and obesity may unravel innovative therapeutic strategies in people affected by obesity-linked insulin resistance-obesity.

Key Words: Vaspin-insulin resistance-obesity.

Introduction

THE incidence of obesity is worldwide increasing and its development seems to be as a result of high-caloric diet intake and physical inactivity [1]. In fact one of the critical determinants for the development of obesity associated with subsequent complications may be an increase in the regional distribution of body fat i.e. abdominal obesity. The latter has been linked to significant metabolic abnormalities, including insulin resistance, hyper-insulinemia, and elevated triglyceride levels, as well as increased incidence of hypertension, glucose intolerance, diabetes mellitus and an increase risk of cardiovascular diseases [2,3].

It has been hypothesized that large amounts of visceral fat cause insulin resistance because lipolysis of visceral adipose tissue triglycerides releases free fatty acids (FFAs) directly into the portal vein, which are then transported to the liver. Increased delivery of FFAs to the liver impairs insulin’s ability to suppress hepatic glucose production, and increased systemic FFAs concentration inhibits insulin-mediated glucose disposal in skeletal muscle [4]. However, data from studies that examined FFA kinetics in human revealed that FFA released from visceral fat represent only a small percentage of total FFA delivered to liver and muscle tissues [5].

Scientists and physicians no longer view adipose tissue as just an endocrine target but now appreciate it as an endocrine organ in its own right [6]. Mature adipocytes secrete a class of proteins, known as adipokines. These include adiponectin [7], leptin [8], plasminogen activated inhibitor-1 (PAI-1) [9] and resistin [10]. These discoveries have been the impetus for a more thorough and comprehensive analysis of the secreted proteins derived from adipocytes.

Vaspin is a recently identified visceral adipose tissue-derived serine protease inhibitor (serpin). Its cloned DNA was first isolated from visceral white adipose tissues (WATs) of fatty obese rats with type 2 diabetes at the peak of their insulin resistance [11]. However, the correlation between
vaspin and markers of insulin sensitivity and obesity is still controversial. Seeger, et al. [12] found no correlation between vaspin and insulin sensitivity in diabetic patients. Still very little is known about its production by abdominal fat and its relation to the metabolic abnormalities associated with obesity.

The aim of this study was to:
1. Assess vaspin mRNA expression in abdominal subcutaneous and visceral adipose tissue of diet-induced obese rats.
2. Evaluate whether vaspin is correlated with the visceral fat weight, insulin resistance and serum leptin level in these obese rats.
3. Investigate the effect of 3 weeks exercise on abdominal adipose tissue vaspin expression in relation to the previous parameters.

Material and Methods

Animals and experimental design:
Animals were purchased from the animal care unit of Cairo Medical University, all procedures that involved animals were approved by this unit. Male wistar rats, 8-10 weeks old, weighing 150-170 g, were housed each in a cage in a constant temperature (22-24°C) and light controlled room on an alternating 12:12 h light-dark cycle and had free access to food and water [13].

Animals were divided into the following groups:
1. Control group (n=7): Receiving standard chow for 10 weeks (6.5% Kcal fat).
2. Diet-induced obese group (DIO) (n=15): Receiving the standard chow with the addition of 100 g/Kg of butter fat for 10 weeks as previously prepared by Ward and Coates [14].
3. Control+Exercise group (C+EXE) (n=8): Receiving standard chow for 10 weeks (6.5% Kcal fat) concomitant with the performance of swimming exercise daily in the last 3 weeks.
4. DIO+EXE group (n=15): Receiving the standard chow with the addition of fat (as in DIO group) for 10 weeks, concomitant with the performance of swimming exercise daily in the last 3 weeks.

Exercise-training regimen:
Rats initially swam 15 min/day (6 days/wk) in a temperature-controlled bath set at 36°C, and duration was gradually increased by 15 min/wk until a regimen of 45 min/day was achieved. Training lasted for 3 weeks. During the swimming exercise, four animals swam together in a plastic barrel measuring 60 cm in diameter and filled to a depth of ~60 cm. Group swimming was chosen because rats usually climb over each other, and in this way more vigorous muscle activity is achieved than when animals are allowed to swim alone. After that, they were dried and placed in their cages [15,16].

Blood pressure measurement:
The systolic blood pressure (SBP) was measured under conscious conditions at the beginning of the experiment and after 1, 3, 6 and 10 weeks of the diet, to monitor any diet-induced changes in the blood pressure. Before measuring the systolic blood pressure, the body temperature of the rats had to be adjusted to 37°C using an incubator for 10 min. The body temperature was verified by colonic temperature measurement. The SBP was assessed with the tail-cuff method using the electrosphygmomanometer. As normal blood pressure shows intrinsic diurnal variation and may be disturbed by environmental conditions, all measurements were carried out between 09:00 and 10:00 a.m. in a quite room. The average of 3 pressure readings was recorded for each measurement [17].

Blood and tissue samples:
After 10 weeks, rats were weighed and retroorbital blood samples were taken from the rats of all groups after an overnight fasting, to measure fasting serum glucose, insulin, triglycerides (TG) and leptin levels.

Insulin resistance index HOMA-IR calculation:
The homeostasis model assessment of insulin resistance (HOMA-IR) was used as a measure of insulin resistance [HOMA index= fasting glucose (mmol/l) x insulin (pmol/l)/ 22.5] [18]. β-cell function is given by HOMA-β = (3.08 x fasting insulin)/(fasting glucose-3.5) [19].

Fat samples:
After the collection of the blood samples, the rats were decapitated and the fat pads from three visceral fat regions (epididymal, retroperitoneal + perirenal, and mesenteric + omental) were dissected and weighed as visceral fat weight [20]. Sample was taken from the abdominal subcutaneous (inguinal fat depot) and all the visceral fat depots were homogenized for assessment of vaspin gene expression.

Measurement of insulin:
Non-radioactive quantification of insulin in rat serum was done using RAT/MOUSE insulin ELISA kit (Linco, Millipore USA) according to manufacture’s instructions [21].
Measurement of Leptin:
Non-radioactive quantification of Leptin in rats’ sera was done using Rat/ MOUSE Leptin ELISA (Biovendor, USA) as described previously [22].

Detection of vaspin gene expression:
• RNA extraction:
  Total RNA was extracted from visceral and subcutaneous adipose tissue samples, using a commercially available acid-phenol reagent (TRIzol, Invitrogen Corp.). A set concentration of RNA was reverse transcribed into cDNA by using reverse transcriptase and random hexamer primers as described in the manufacturer’s protocol (Invitrogen Corp.).

• The reverse transcription -polymerase chain reaction (RT-PCR):
  Quantitative PCR of vaspin gene was performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany). PCRs were carried out in a reaction mixture consisting of 5.0 µl reaction buffer and 2.0 mmol/l MgCl₂ (Biogene, Kimbolton, U.K.), 1.0 µl of each primer (10 ng/µl), 2.5 µl cDNA, and 0.5 µl Light Cycler DNA Master SYBR Green I (Roche). Protocol conditions consisted of denaturation at 95ºC for 15 s, followed by 40 cycles of 94ºC for 1 s, 58ºC for 10 s, and 72ºC for 12 s. Quantitative amounts of vaspin gene were standardized against the housekeeping gene β-actin. Rat visceral adipose tissue specific SERPIN (Vaspin) primers were: Forward 5’-AGTCGG AAAACCCACAACAG-3’ and Reverse 5’-ATGGTGCAAGGAGCTGACT-3’ and The sequence of the primers of β-actin was F: 5’TCA CCCCATTACCCTGGAG3’ R: 5’TTCG CTTTTGTTTCAGGGG-3.’

  All the PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and visualized by UV transilluminator. The PCR products were semi-quantitated using the densitometer system, (BioDoc Analyze, Biometra, Germany) [23].

Western blotting:
Subcutaneous and visceral fats were homogenized in radioimmuno-precipitation lysis buffer (Upstate, Lake Placid, NY) according to the manufacturer’s instructions. Protein samples (30 µg/lane) containing SDS-sample buffer (5 mol/l urea, 0.17 mol/l SDS, 0.4 mol/l dithiothreitol, and 50 mmol/l Tris-HCl, pH 8.0) were subjected to SDS-polyacrylamide gel electrophoresis (10% resolving gel) and transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary rabbit-anti-human antibody for vaspin (Phoenix Pharmaceuticals, Burlingame, CA) (1:1,000 dilution) or primary rabbit-anti-human antibody for β-actin (Cell Signaling Technology, Beverly, MA) (1:1,000 dilution) left overnight at 4ºC. The membranes were washed thoroughly for 60 min with TBS/0.1% Tween before incubation with the secondary anti-rabbit horseradish peroxidase-conjugated Ig (Dako, Ely, Cambridgeshire, U.K.) (1: 2,000) for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (ECL, Amersham Biosciences, Arlington Heights, IL). Band intensity was determined using the Versa Doc 5000 Imaging system (Bio-Rad Laboratories, Hercules, CA) [24].

Statistical analysis:
The results were analyzed using SPSS computer software package, version 10.0 (Chicago-IL, USA). Data were presented as mean ± S.D.

Differences among the parameters of the different groups were compared by one-way ANOVA and Bonferroni post-hoc test. Association between visceral vaspin positive expression in DIO and DIO+EXE groups was tested by Chi-square test (X²). The data for HOMA-IR and HOMA beta cell function (HOMA -β) were transformed logarithmically before analysis [25]. Pearson correlation was used to correlate vaspin expression to the different measured parameters. The results were considered statistically significant at p≤0.05.

Results
Effect of high-fat diet and exercise on body weight, visceral fat weight and SBP:
Body weight and systolic blood pressure were insignificantly different between groups at the beginning of the study (p>0.05).

As revealed in table (1), feeding rats high-fat diet for 10 weeks, significantly increased body weight, visceral fat weight and systolic blood pressure in the DIO group compared to the control one (p≤0.05). Swimming for 3 weeks had no effect on these parameters in the C+EXE compared to control group, and still the DIO+EXE group had a significantly elevated body and visceral fat weights (p<0.001) but without a significant change in SBP compared to C+EXE group. Meanwhile, exercise improved the systolic blood pressure in DIO+EXE group compared to DIO group without significant change in both body and visceral fat weights.
Table (1): Effect of high fat diet and exercise on metabolic parameters in the studied groups.

<table>
<thead>
<tr>
<th>Measured parameters</th>
<th>Control group</th>
<th>DIO group</th>
<th>C+EXE group</th>
<th>DIO+EXE group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=7</td>
<td>n=15</td>
<td>n=8</td>
<td>n=15</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>180.7±1.7</td>
<td>211.6±11.6*</td>
<td>181.9±3.6</td>
<td>209.5±9.2#</td>
</tr>
<tr>
<td>Visceral Fat weight (g)</td>
<td>2.347±1.124</td>
<td>5.321±1.480*</td>
<td>2.003±0.490</td>
<td>4.870±1.517#</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>121.7±4.8</td>
<td>136.9±8.1*</td>
<td>131.0±2.45</td>
<td>126.8±7.9+</td>
</tr>
</tbody>
</table>

*: Significant compared to control group. +: Significant compared to DIO group. #: Significant compared to C+EXE group.

**Effect of high-fat diet and exercise on parameters of insulin resistance and serum leptin:**

As shown in table 2 and Fig. (1: A&B), DIO rats had significantly increased fasting serum insulin, TG and leptin levels as well as HOMA-IR compared to control rats with no statistical significant differences in fasting serum glucose and in HOMA-ß, indicating that DIO rats had developed insulin resistance.

Swimming exercise for 3 weeks was able to significantly reduce the fasting serum glucose, and insulin as well as the HOMA-IR in C+EXE compared to the control group (p≤0.05).

Still the DIO+EXE group had a significantly elevated serum insulin and leptin levels as well as HOMA-IR and HOMA-ß compared to C+EXE group.

However, exercise significantly reduced serum glucose, insulin, TG and leptin levels and improved both HOMA-IR and HOMA-ß in DIO+EXE group compared to the DIO group (Table 2, Fig. 1: A&B). These findings highlight the beneficial effect of exercise.

Table (2): Effect of high-fat diet and exercise on insulin resistance parameters and serum leptin in the studied groups.

<table>
<thead>
<tr>
<th>Measured parameters</th>
<th>Control group</th>
<th>DIO group</th>
<th>C+EXE group</th>
<th>DIO+EXE group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=7</td>
<td>n=15</td>
<td>n=8</td>
<td>n=15</td>
</tr>
<tr>
<td>Fasting serum glucose (mmol/l)</td>
<td>5.5±0.5</td>
<td>5.7±0.6</td>
<td>4.6±0.5*</td>
<td>4.1±0.7+</td>
</tr>
<tr>
<td>Fasting serum insulin (pmol/l)</td>
<td>238.6±52.5</td>
<td>445.3±71.0*</td>
<td>158.6±26.7*</td>
<td>316.1±43.9+#</td>
</tr>
<tr>
<td>Serum TG (mg/dl)</td>
<td>69.04±3.79</td>
<td>89.61±4.31*</td>
<td>68.41±3.84</td>
<td>68.78±3.65+</td>
</tr>
<tr>
<td>Fasting serum leptin (ng/ml)</td>
<td>2.36±0.36</td>
<td>29.67±2.69*</td>
<td>1.82±0.18</td>
<td>23.13±3.33+#</td>
</tr>
</tbody>
</table>

*: Significant compared to control group. +: Significant compared to DIO group. #: Significant compared to C+EXE group.

![Insulin resistance index (HOMA-IR) (A) and HOMA beta cell function (HOMA-ß) (B) in the studied groups.](image-url)
Expression of vaspin:

The effect of fat rich diet, exercise and both of them on vaspin gene expression was examined in both the abdominal subcutaneous and visceral adipose tissues. No vaspin mRNA expression was detected in abdominal subcutaneous and visceral adipose tissues from control and C+EXE rats while it was variably expressed in DIO and DIO+EXE rats with higher expression in visceral than abdominal subcutaneous adipose tissue ($p<0.05$), suggesting that transcription of vaspin is induced by obesity and not by exercise and its primary site is the visceral adipose tissue.

As revealed in Fig. (2,3) vaspin was expressed in the abdominal subcutaneous tissue of only 2 cases out of 15 (13.3%) for each of the DIO and DIO+EXE groups, however, in visceral adipose tissue, vaspin mRNA was detected in DIO rats in 6 cases out of 15 (40%) including the 2 cases that expressed vaspin in the subcutaneous tissue. While in DIO+EXE rats, it was expressed in 4 cases out of 15 cases (26.7%) including also the 2 cases that expressed vaspin in the subcutaneous tissue (Fig. 3).

Meanwhile, table 3 shows that compared to DIO rats, DIO+EXE group showed no significant difference in the expression of both subcutaneous and visceral vaspin ($X^2=0.6, p>0.05$).

The changes noted at the mRNA level were also reflected at the protein level in DIO and DIO+EXE groups (Fig. 4).

Univariate correlations:

Visceral vaspin expression in the DIO group was positively correlated with body weight ($r=0.869, p<0.05, n=6$) and HOMA-IR ($r=0.881, p<0.001, n=6$). No correlation was found between vaspin and serum leptin, HOMA-β, SBP or visceral fat weight.

![Lane M: Molecular DNA marker (Ø× 174 Hae III). Lanes 1-2: PCR products of Vaspin mRNA in subcutaneous fat of DIO rats. Lanes 3-8: PCR products of Vaspin mRNA in visceral fat of DIO rats.](image1)

![Fig. (2): Agarose gel electrophoresis 2% stained with ethidium bromide showing PCR products of vaspin mRNA in subcutaneous and visceral fats of obese rats ((n=2 and n=6 respectively).](image2)

![Lane M: Molecular DNA marker (PCR marker). Lanes 1-2: PCR products of Vaspin mRNA in subcutaneous fat of DIO+EXE. Lanes 3-6: PCR products of Vaspin mRNA in visceral fat of DIO+EXE.](image3)

![Fig. (3): Agarose gel electrophoresis 2% stained with ethidium bromide showing vaspin mRNA products in subcutaneous and visceral fats of obese rats after exercise (n=2 and n=4 respectively).](image4)

<table>
<thead>
<tr>
<th>Groups</th>
<th>DIO (n=15)</th>
<th>DIO+EXE (n=15)</th>
<th>$X^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral fat depot vaspin expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>6 (40%)</td>
<td>4 (26.7%)</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

It is now well acknowledged that the consequences of obesity, particularly insulin resistance, are influenced to a great extent by the actions of adipokines. Many studies support the key role of some of these adipokines in the development of obesity-related disorders and the metabolic syndrome, particularly in the pathogenesis of type 2 diabetes [26]. In this study, the recently identified adipokine, vaspin mRNA was expressed in the obese and not in the control group positively correlated with the body weight, indicating that vaspin expression is related to obesity. Moreover it was more frequently detected in the obese rat abdominal visceral fat compared to the subcutaneous one (40% versus 13.3%, \( p < 0.05 \)). Thus it can be suggested that the visceral fat is the major and not the only site for the synthesis of vaspin in obesity.

Visceral and subcutaneous adipocytes have different capacities to produce hormones and enzymes [27]. Such fat depot differences in protein or mRNA expression have been reported in other studies for several adipokines. Vaspin could be one of them. It is possible that fat cells in various regions have different origins and, because of this, express different genes [28].

Similar to our results, Kloting, et al. [29] have shown that vaspin mRNA expression was more frequently detectable in human visceral fat (23%) than abdominal subcutaneous fat (15%), but those findings were detected in patients with type 2 diabetes. Also in their work, visceral vaspin expression significantly correlated with body mass index (BMI) and % body fat whereas vaspin mRNA expression was not detectable in lean subjects.

In contrast, the same authors two years latter [30] found no correlation in patients with type 2 diabetes, between circulating vaspin and BMI. They suggested either that a dysregulation of vaspin secretion occurs in patients with type 2 diabetes or that diet and metformin therapy of type 2 diabetes might contribute to the lack of association between obesity and increased circulating vaspin.

Meanwhile, Wada [31] found vaspin mRNA expression exclusively in visceral white adipose tissue (WATs) of Otsuka Long-Evans Tokushima fatty rat, an animal model of abdominal obesity with type 2 diabetes, at the peak of their body weight and insulin resistance.

Visceral fat could cause metabolic abnormalities by secreting inflammatory adipokines, such as interleukin-6, tumor necrosis factor- \( \alpha \), macrophage chemoattractant protein-1, and resistin, which induce insulin resistance and diabetes [32,33]. In contrast, visceral fat might have beneficial metabolic effects by producing adiponectin, which increases insulin sensitivity and decreases glucose intolerance and diabetes [34].

In the present study, DIO group shared common components of human obesity, including the significant increase in body weight, visceral fat weight, SBP, serum insulin, TG and leptin compared to the control group. Vaspin was expressed in DIO abdominal visceral fat when the insulin sensitivity was reduced (+ve correlation between vaspin and HOMA-IR) however independent from the HOMA-\( \beta \) cell function. These results may imply a role for vaspin in the obesity-induced insulin resistance.

Similarly, Hida and co-workers [11] characterized vaspin as an interesting novel adipokine with insulin-sensitizing effects. They demonstrated in their mice model of obesity with type 2 DM, hypertension and dyslipidemia, that vaspin expression in visceral adipose tissues and its circulating form
increased at the peak of obesity and insulin resistance. This may provide a plausible explanation for the absence of vaspin expression in some of the DIO group rats in the present study which did not reach the peak of insulin resistance.

These authors also proved that recombinant human vaspin administered to their mice model significantly improved insulin sensitivity and glucose tolerance and suppressed the expression of leptin, resistin, and TNF-α, whereas it increased that of the glucose transporter-4 and adiponectin in WATs. As this response was absent in lean mice, they suggested that vaspin modulates insulin action conceivably only in the presence of its target proteases in WATs.

Li, et al. [35] suggested that the increase in vaspin may be a compensatory response to antagonize the action of other unknown proteases that are up-regulated in obesity and in states of insulin resistance, hence, this up-regulation may be a defensive mechanism against insulin resistance.

Moreover Tan, et al. [36] reported increased circulating vaspin levels as well as increased expression of vaspin mRNA and protein levels in omental adipose tissue of overweight women with polycystic ovary syndrome positively related to serum glucose level and HOMA-IR but independent from HOMA-β function.

Furthermore, Gonzalez, et al. [37] demonstrated that vaspin expression was minimal after fasting in non-obese rats and its level was partially increased after leptin treatment. They suggested that WAT vaspin mRNA expression is regulated by nutritional status, and leptin seems to be the nutrient signal responsible for those changes.

However, Seeger, et al. [12] failed to find a correlation between vaspin levels and markers of insulin sensitivity and glucose metabolism, including fasting glucose, fasting insulin, (HOMA), and adiponectin in chronic haemodialysis patients and control subjects, which included diabetic patients.

In the present study, after 3 weeks of a swimming exercise regimen without change in diet content, C+EXE group showed no vaspin expression similar to control group. Moreover, DIO+EXE rats showed no significant change in visceral vaspin expression compared to the DIO, despite the significant reduction in HOMA-IR as well as significant improvements of other parameters of insulin resistance and HOMA-β-cell function. These results denote that vaspin expression is not affected by exercise and remains as a marker of the increase in body weight which was still evident in the DIO+EXE group.

These speculations are supported by findings of Hida, et al. [11] that running wheel exercise suppressed vaspin mRNA expression in visceral white adipose tissues of obese diabetic rats when the body weight decreased.

In contrast, Kloting, et al. [30] found that although vaspin serum concentrations were lower in lean subjects compared to obese ones, they increased in both groups with weight loss associated with a 4 weeks of intensive physical training program. In contrast, vaspin serum level was unchanged in trained athletes undergoing the same regimen. The authors suggested that vaspin serum concentration is differentially regulated in the resting state and after exercise and might represent a transient adaptation mechanism. They speculated that increased vaspin serum concentrations contribute to the insulin-sensitizing effects of physical activity.

In conclusion, the data of the present study demonstrate that visceral vaspin is expressed in obesity associated with insulin resistance and unchanged by physical training unaccompanied by decrease in body weight. These findings are relatively preliminary, but may spark future research to establish this adipokine as a marker of obesity and as an anti-insulin resistance treatment.

References


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