Glucose and insulin modify thrombospondin 1 expression and secretion in primary adipocytes from diet-induced obese rats

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ABSTRACT

Thrombospondin 1 (TSP-1), an anti-angiogenic factor and TGF-β activity regulator, has been recently recognized as an adipokine that correlates with obesity, inflammation and insulin-resistance processes. In the present study, epididymal adipocytes of rats that were fed a chow (C) or a high fat diet (HFD) for 50 days, were isolated and incubated (24-72 h) in low (LG; 5.6 mM) or high (HG; 25 mM) glucose, in presence or absence of 1.6 nM insulin. Rats fed the HF diet showed an established obesity state. Serum TSP-1 levels and TSP-1 mRNA basal expression of adipocytes from HFD rats were higher than those from controls. Adipocytes from HFD animals presented an insulin-resistance state, as suggested by the lower insulin-stimulated glucose uptake as compared to controls. TSP-1 expression in culture was higher in adipocytes from obese animals at 24 h, but when the adipocytes were treated with HG, these expression levels dropped dramatically. Later at 72 h, TSP-1 expression was lower in adipocytes from HFD rats, and no effects of the other treatments were observed. Surprisingly, the secretion levels of this protein at 72 h were increased significantly by the HG treatment in both types of adipocytes, although they were even higher in adipocytes from obese animals. Finally, cell viability was significantly reduced by HG treatment in both types of adipocytes. In summary, TSP-1 expression/secretion was modulated in an *in vitro* model of insulin-resistant adipocytes. The difference between expression and secretion patterns suggests a post-transcriptional regulation. The present study confirms that TPS-1 is closely associated with obesity-related mechanisms.

Keywords: diet-induced obesity, adipokine, cell culture, adipose tissue

INTRODUCTION

An excessive body fat accumulation could drive to several associated clinical complications such as type 2 diabetes, metabolic syndrome features, cardiovascular diseases, inflammatory disturbances, etc [8]. These manifestations have been linked to an impaired production and secretion of endogenous products by the enlarged adipocytes or the accompanying macrophages from the white adipose tissue (WAT) stroma-vascular fraction [8]. Actually, several inflammatory products derived from this tissue (TNF-α, IL-6, MCP-1, iNOS) have shown positive associations with body adiposity [16]. Besides the pro-inflammatory molecules secreted by WAT, this tissue also produces numerous adipokines that may have also important local and systemic effects [15].

Thrombospondin 1 (TSP-1) is a molecule that was first identified as a thrombin-sensitive protein released upon activation of platelets by thrombin [6]. It was initially isolated from platelets and megakaryocytes [28], but was later detected in several cell types such as macrophages and adipocytes [21, 22, 30, 32]. Nowadays, it is known that TSP-1 is a multifunctional protein composed by multiple structural domains [5, 10], and it has been especially related with several anti-angiogenic pathways [20, 23, 24]. Recently, this protein has been included among the group of adipokines, since its expression is increased in WAT of obese and insulin-resistant subjects, presenting positive correlations with the level of adiposity [35, 41]. Furthermore, it has been reported as an important factor in the adipocyte- and macrophage-driven inflammation in the adipose tissue, and that could mediate the elevation of PAI-1, promoting a prothrombotic state [41]. Also, TSP-1 has been described as a major regulator of the transforming growth factor (TGF)-β activity [11, 31], which, together with the increased

PAI-1 levels, are correlated with different features of obesity, insulin-resistance, and metabolic syndrome [1, 29, 37]. Finally, it has been described higher TSP-1 gene expression in visceral respect to subcutaneous adipose tissue in obese subjects [35].

Moreover, it has been described that the expression of TSP-1 in blood vessels is increased in diabetes, perhaps by a direct effect of the elevated glucose levels in blood [39]. Nevertheless, no effects of glucose incubation have been studied so far in adipocytes from obese specimens. Thus, the purpose of this study was to characterize TSP-1 expression and secretion patterns in adipocytes isolated from either lean or diet-induced obese rats, in order to demonstrate that this important protein is involved in the modulation of the inflammatory processes occurring in WAT.

MATERIALS AND METHODS

Materials

Dulbecco's modified eagle's medium (DMEM), 100X minimal essential medium (MEM) non-essential amino acids, penicillin/streptomycin, heat-inactivated fetal bovine serum (FBS), 10X MEM, nystatin, Trizol Reagent, and M-MLV reverse transcriptase were obtained from Invitrogen (Paisley, UK). Bovine serum albumin (BSA), HEPES, insulin and 2′,7′-dichlorofluorescein were all obtained from Sigma-Aldrich Company (St. Louis, USA). Collagen (Purecol) was purchased from Nutacon (Leimuiden, The Netherlands). Type I collagenase was supplied by Worthington Biochemical Corporation (Lakewood, USA). Glycerol-3-phosphate dehydrogenase (GPDH), glycerol kinase (GK), adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide (NAD) were obtained from Roche Diagnostics (Mannheim, Germany).

Animals and dietary treatment

Eight-week-old male Wistar rats (n=20) supplied by the Center for Applied Pharmacobiology Research (CIFA, Pamplona, Spain), were housed in a temperature-controlled room at 21-23 ºC with a 12 h light cycle (lights goes off at 8 pm). The animals were assigned into two different dietary groups: a control group (n=10) that was fed a standard chow diet (2014 Tekland Global 14% Protein Rodent Maintenance Diet; Harlan Iberica, Barcelona, Spain) containing 16.6% of energy as protein, 73.1% of energy as carbohydrate (7% as simple sugars) and 10.3% of energy as lipid by dry weight, and a high fat diet-fed group (HFD, n=10) that was fed a high fat diet (D12330 diet, 58 kcal% fat w/cornstarch Surwit Diet; Research Diets Inc., New Brunswick, USA) containing 16.4% of energy as protein, 25.5% of energy as carbohydrate (4.5% as

simple sugars) and 58.0% of energy as lipid by dry weight, as previously described [3]. Both groups of animals had *ad libitum* access to water and food during the trial, being their body weight and food intake daily recorded. After 50 days of dietary treatment the animals were euthanized. The final body weights were recorded and blood and tissue samples of several WAT depots were immediately collected and weighed. About 3-4 g of epididymal WAT were immediately isolated from total tissue for adipocyte isolation and primary culture experiments. All the procedures were performed according to European, national and institutional guidelines of the Animal Care and Use Committee at the University of Navarra.

Serum measurements

The circulating glucose levels were measured with the HK-CP kit (ABX diagnostic, Montpellier, France) using an automatized COBAS MIRA equipment (Roche, Basel, Switzerland). Serum insulin (Mercodia AB, Uppsala, Sweden), TSP-1 (Uscn Life Science Inc., Wuhan, China), and leptin (Linco Research, St. Charles, USA) levels were determined by different ELISA kits, using an automatized Triturus equipment (Grifols International, Barcelona, Spain).

Adipocyte isolation

Primary cultures were obtained according to protocols described elsewhere [18]. Epididymal WAT (3-4 g) of each rat was minced with scissors in HEPES-phosphate buffer (pH 7.4; containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2.2 mM $CaCl_2 \cdot 2H_2O$, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4, and 10 mM HEPES). WAT fragments were digested in the same buffer with type I collagenase (1.25 mg/ml per 0.5 g tissue) at 37°C with gentle shaking for 30 min. The resulting cell

suspension was diluted in the buffer, and then the adipocytes were isolated from the undigested tissue by filtration through a 400 μm nylon mesh and washed three times with alternated centrifugations at 500 rpm for 6 minutes. Isolated adipocytes were then resuspended in DMEM supplemented with 1% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, 100 U/ml nystatin and with 1X MEM non-essential amino acids, followed by incubation for 40 min at 37°C in 5% CO_2. The resulting isolated adipocytes (150 μl of a 2:1 mix of packed cells/culture medium) were then plated on 500 μl of a collagen matrix (pH 7, 7 parts collagen : 1 part 10X MEM) in six well's culture plates and at 37°C / 5% CO_2.

Isolated adipocytes measurements

Before primary culture, some measures on the isolated adipocytes were performed. In the first instance, intracellular ROS concentrations were determined using 2′,7′-dichlorofluorescein (DCFH) according to a protocol described elsewhere [17]. Once inside the cell, this molecule is cleaved by endogenous esterases and can no longer pass out of the cell membrane. The de-esterified product becomes a fluorescent compound after oxidation by ROS [7]. Briefly, cells were incubated with 10 μM DCFH for 40 minutes in 37 ºC / 5% CO_2, frozen for at least 1 h at -80ºC and then lysed with 500 μl of lysis buffer (150 mM NaCl, 0.1% Triton and 10 mM Tris). Then, 200 μl of each lysate was plated on a 96-well black plate (Labsystems, Barcelona, Spain). Finally, fluorescence intensity was measured with a POLARstar spectrofluorometer plate reader (BMG Labtechnologies, Offenburg, Germany), at an excitation of 485 nm and an emission of 530 nm wavelengths.

Also, morphologic characteristics of the isolated adipocytes were analyzed. Cells were placed on a Neubauer chamber and coupled on an Olympus CK30 microscope

(Olympus Corporation, Barcelona, Spain). Then, pictures were taken with an Olympus C-5060WZ Digital Camera (Olympus Corporation, Barcelona, Spain) using a Camedia Adapter C5060-ADUS for IUS Microscope (Olympus Corporation, Barcelona, Spain) at 40X magnification. Pictures in TIFF format with 400 x 300 pixels resolution were processed with Scion Image for Windows software (Scion Corporation, Maryland, USA) for cell number and diameter determinations. Finally, mRNA expressions of TSP-1 and leptin in these isolated adipocytes were also measured according to the protocol described below (gene expression assays section).

Adipocytes culture

After the final 40-50 min incubation, the adipocytes were cultured with high (HG, 25 mM; n=5) and low (LG, 5.6 mM; n=5) glucose concentrations, in presence or absence of 1.6 nM insulin. Cells were maintained in an incubator at 37°C in 5% CO_2. Aliquots of the culture medium, and culture plates were collected at 24 h and at the end of the experimental trial (72 h) and frozen at -80 ºC for further assays.

Culture medium determinations

All the assays were performed on 24 and 72 h samples. Glucose and lactate concentrations in the medium were measured with the HK-CP kit from ABX diagnostic (Montpellier, France) and with the L-lactate kit from Randox Laboratories (Crumlin, UK), respectively, using an automatized COBAS MIRA equipment (Roche, Basel, Switzerland). The cellular glucose uptake was estimated by the difference between the content of glucose in the culture medium at the beginning and at the end of the experiment (24- 72 h), and the lactate value detected in the medium corresponds to the lactate production by the cells after the trial. Increasing glycerol concentration in the

medium after 24 or 72 h, as a measure of the adipocyte lipolytic response, was determined by a colorimetric method [9]. Briefly, culture medium of each sample was incubated with 25 μg/ml GPDH and 250 mU/ml GK, in the presence of 43.6 mM $MgCl_2$, 200 mM glycine, 5.2 % hydrazine, 1.24 mM ATP and 573 μM NAD for 40 minutes. Then, samples absorbance was measured at 340 nm in a microplate reader (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, USA). Finally, TSP-1 secretion at 72 h was determined by the ELISA kits as previously indicated in the serum measurements section.

Gene expression assays

Total RNA was isolated from isolated epididymal adipocytes using Trizol (Invitrogen, Paisley, UK) according to the manufacturer protocol. Purified total RNA from adipocytes were then treated with DNAse (DNAfree kit; Ambion Inc., Austin, USA) and used to generate cDNA with M-MLV reverse transcriptase (Invitrogen, Paisley, UK). Relative real-time PCR was performed on an ABI PRISM 7000 HT Sequence Detection System (Applied Biosystems, California, USA). Taqman probes for rat leptin (Rn00565158_m1) and TSP-1 (Rn01513690_m1) mRNA and 18S (Hs99999901_s1) rRNA, were also supplied by Applied Biosystems (California, USA). All the expression levels of the target genes studied were normalized by the expression of the selected internal control, 18S. All procedures were performed according to protocols described elsewhere [18].

Cell viability assay

The LDH activity (μU/ml) in culture medium was used as an indicator of cell membrane integrity, thus as a measurement of cells necrosis/apoptosis [33]. Cell

viability was measured with the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit according to manufacturer indications (Cayman Chemical Company, Ann Arbor, USA), and was assayed at the end of the experimental period (72 h) in all treatments.

Statistical analyses

For body, biochemical and isolated adipocytes-related measurements that were performed before the primary culture, Student t tests were used for analyzing differences among experimental groups. For primary culture samples, two-way ANOVA was performed. For association analyses, the Pearson correlation coefficient was calculated. All results are expressed by mean ± standard error of the mean. A probability of $p<0.05$ was set as statistically significant. All the analyses were performed using the SPSS 15.0 for Windows software (SPSS Inc., Chicago, USA).

RESULTS

High fat diet-induced obesity

The high-fat dietary treatment effectively induced overweight in the animals, as evidenced by the elevated body and WAT weights, and higher levels of some biochemical blood markers, such as glucose, insulin, and leptin, that were observed in rats fed the HFD as compared to rats fed the chow diet (Figures 1A and 1B). TSP-1 blood levels in obese animals were slightly although not significantly higher than those found in the controls (Figure 1B). Also, the adipocytes isolated from epididymal WAT of HFD-fed rats presented different morphological and functional characteristics as compared to the adipocytes from chow-fed rats, such as higher cell diameter and intracellular ROS content, higher leptin gene expression and lower cell number (Figure 1C). TSP-1 gene expression was significantly higher in adipocytes from obese than from control rats (Figure 1C).

Furthermore, positive correlations were found between TSP-1 blood levels and retroperitoneal WAT weight ($r = 0.676$, $p<0.05$) and insulin blood levels ($r = 0.671$, $p<0.05$), and between gene expression levels of TSP-1 and leptin in epididymal WAT ($r = 0.615$, $p<0.05$).

Biochemical measurements in adipocyte culture

Glucose uptake, lactate production and glycerol release of isolated adipocytes from both groups of animals were determined after 24-72 h of treatment (Figure 2). The glucose uptake was decreased in adipocytes from obese animals respect to controls (Figure 2A). At 24 h, only the adipocytes from control animals presented positive responses toward insulin and HG treatments (Figure 2A). At 72 h, insulin induced glucose uptake only in

adipocytes from control animals, and HG treatment inhibited the glucose uptake in both types of adipocytes (Figure 2A). Regarding lactate production, the release of this metabolite to culture medium was reduced in adipocytes from obese rats (Figure 2B). At 24 h, insulin induced lactate release only in adipocytes from control animals, but it affected both types of adipocytes at 72 h (Figure 2B). HG treatment produced no significant effects. Finally, concerning glycerol release, once again the previous HFD treatment induced lower rates of glycerol liberation respect to controls (Figure 2C). No effect was observed by insulin treatment, and a marginal effect of HG was shown at 24 h in adipocytes from obese animals (Figure 2C).

TSP-1 gene expression and secretion in culture

The dietary treatment induced important differences in expression and secretion of TSP-1 (Figures 3A and 3B). At 24 h, TSP-1 gene expression was significantly higher in adipocytes from obese animals than in those from control rats (Figure 3A). When the cells were incubated with HG, no differences were observed in the expression of this protein in control adipocytes. However, the TSP-1 mRNA expression in adipocytes from obese rats presented a drastic decrease (Figure 3A). At 72 h, TSP-1 gene expression was lower in HFD adipocytes, and the insulin treatment inhibited the expression of this protein in both types of cells (Figure 3A). On the other hand, and normalizing 24-72 h data to 0h for taking into account the overall effect of the culture, the number of TSP-1 mRNA copies significantly decreased with time, except for the adipocytes from HFD-LG group (data not shown).

Finally, concerning the secretion of this molecule at 72 h, it was higher in adipocytes from obese animals (Figure 3B). Interestingly, TSP-1 secretion was induced when the

cells were incubated with HG (Figure 3B) in opposition to that found for the gene expression analysis. This effect was observed in both types of adipocytes.

Cell viability analysis

The cell integrity of the cells in culture was evaluated at the end point of the experiment (72 h) (Figure 4). No significant effects were induced neither by the dietary treatment nor the insulin incubations. However, the HG treatment significantly reduced cell viability in both types of adipocytes.

DISCUSSION

In the present report, a nutrigenomic analysis of TSP-1 in insulin-resistant adipocytes under several obesity-related conditions was performed. The HFD-fed rats utilized in this work showed an obesity state [4] that correlated positively with the TSP-1 levels in blood, and with the mRNA expression of this protein in adipocytes from epididymal WAT, which is consistent with a previous report [41]. Interestingly, when the adipocytes were subjected to culture treatments, cells from HFD rats showed lower TSP-1 gene expression than controls, except when the adipocytes where cultured under LG conditions. Also, gene expression pattern differed drastically from the protein secretion levels under HG conditions, being inhibited and induced by this treatment, respectively. These phenomena could be attributed to post-translational mechanisms [2]. Several functions have been attributed to TSP-1 including antiangiogenic [14, 38] and proatherogenic [36, 40] properties. More recently, this protein has been studied as a potential link between vasculature mechanisms and hyperglycemia [2, 39]. An increase in TSP-1 production has been related with atherogenesis and restenosis in diabetes, since this increase could be a response of vascular cells to higher levels of glucose [39]. Moreover, in this same study, it was observed that glucose incubations stimulated the expression and secretion of TSP-1 in cultured endothelial cells [39]. This stimulation seems to be mediated by glycosylation of specific nuclear proteins that participate in the hexosamine pathway of glucose catabolism [34]. However, in another report from the same investigation group, a cell-type specific modulation by HG treatment was observed [2]. In such work, the authors described that, unlike in macrovascular cells, TSP-1 levels dropped as a result of the HG treatment in microvascular endothelial cells and retinal pigment epithelial cells. This downregulation was suggested to be post-

transcriptional, since the mRNA levels of TSP-1 were increased. The authors proposed that this post-transcriptional suppression was controlled by untranslated regions of TSP-1. This mechanism would not be present in vascular smooth cells and fibroblasts, where the levels of TSP-1 mRNA expression correlate positively with the increased secretion of this protein as a result of HG exposure [39].

However, these results contradict the findings of the present work, since these authors described higher TSP-1 gene expression and lower TSP-1 secretion upon glucose stimulation. In spite of this, a recent study revealed that, in smooth muscle cells, the difference in the amount of TSP-1 levels upon high or low glucose concentration could be due to a difference in the uptake and degradation of this molecule [26]. Moreover, the authors described that using lysosomal degradation inhibitors it is possible to restore the TSP-1 levels in normal glucose concentration respect to HG treatment, and the same inhibitor treatment in HG treated cells produced no significant increase in TSP-1 protein levels. The authors suggested also that the increase in TSP-1 protein levels in hyperglycemia could be a protection from lysosomal degradation. Furthermore, it has also been described that the TSP-1 mRNA is regulated by a rapid internalization through the endocytic receptor LRP-1 (low density lipoprotein-related protein) leading to its degradation [12, 13, 27]. Other reports have also shown that LRP-1 levels are related with insulin resistance status, since they were inhibited in the aortic arch of diabetic hamsters [42] and in the brain vessels of streptozotocin-induced diabetic rats [19]. All this facts could explain why the degradation of TSP-1 mRNA is higher in presence of HG in this work, and opposed to its secretion in culture. Moreover, this effect is dependent of the dietary history of the adipocytes, being mRNA degradation delayed in cells from obese animals.

This is the first time that TSP-1 modulation is evaluated in adipocytes from obese animals under hyperglycemia conditions. According to the present findings, adipocytes could also contribute to the development of collateral symptoms of diabetes through TSP-1 signaling [25]. This observation is consistent with the fact that in the present results: 1) insulin significantly inhibited TSP-1 gene expression, lowering the secretion levels of this proatherogenic agent, and 2) TSP-1 expression and secretion were augmented in adipocytes from obese animals. Finally, it is worth to mention that it has been reported that TPS-1 exerts antiangiogenic activities in the adipose tissue and modulates the activity of TGF-β and PAI-1, both proteins closely related with insulin-resistance and metabolic syndrome [41].

Summing up, the present study shows that TSP-1 secretion is highly modified in an *in vitro* model that mimics insulin-resistance/obesity conditions, showing a higher response to glucose in adipocytes from rats fed a HF diet. This pattern was opposite to mRNA levels, suggesting a strong post-transcriptional regulation of this gene in adipocytes. The present nutrigenomics study confirms an interaction between diet and glucose in adipose tissue regarding this adipokine and reinforces the idea that TSP-1 is closely related with metabolic obesity-related processes.

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AUTHOR DISCLOSURE STATEMENT

All the authors declare that there are no competing financial interests regarding the contents of this article.

FIGURE LEGENDS

Figure 1. Body, biochemical and adipocyte-related data of rats from both dietary groups. Data (n = 10) are expressed as mean ± standard error of the mean. Student t test was applied for comparisons. *, $p<0.05$; ***, $p<0.001$; n.s., not significant; WAT, white adipose tissue; ROS, reactive oxygen species; HFD, high fat diet.

Figure 2. Glucose and lipid metabolism measurements in the primary culture. Effects of 1.6 nM insulin, and high (25 mM) or low (5.6 mM) glucose concentrations on 24-72 h **(A)** glucose uptake, **(B)** lactate production, and **(C)** glycerol release in adipocytes isolated from rats fed chow (white bars) or high fat (HF) diet (black bars). Data (n = 5) are expressed as mean ± standard error of the mean. Two-way ANOVA was performed. n.s., not significant; INS, insulin; HG high glucose; HFD, high fat diet.

Figure 3. TSP-1 gene expression and protein secretion in the primary culture. Effects of 1.6 nM insulin, and high (25 mM) or low (5.6 mM) glucose concentrations on **(A)** 24-72 h mRNA expression, and **(B)** 72 h protein secretion in adipocytes isolated from rats fed chow (white bars) or high fat diet (black bars). Data (n = 5) are expressed as mean ± standard error of the mean. Two-way ANOVA was performed. n.s., not significant; INS, insulin; HG high glucose; HFD, high fat diet.

Figure 4. Viability assay of the primary culture. Effects of 1.6 nM insulin, and high (25 mM) or low (5.6 mM) glucose concentrations on the 72 h lactate dehydrogenase activity in culture medium in adipocytes isolated from rats fed chow (white bars) or high fat diet (black bars). Data (n = 5) are expressed as mean ± standard error of the mean. Two-way

ANOVA was performed. n.s., not significant; INS, insulin; HG high glucose; HFD, high fat diet.

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