Regulation of visceral adipose tissue-derived serine protease inhibitor by nutritional status, metformin, gender and pituitary factors in rat white adipose tissue

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Abstract

Visceral adipose tissue-derived serine protease inhibitor (vaspin) is a recently discovered adipocytokine mainly secreted from visceral adipose tissue, which plays a main role in insulin sensitivity. In this study, we have investigated the regulation of vaspin gene expression in rat white adipose tissue (WAT) in different physiological (nutritional status, pregnancy, age and gender) and pathophysiological (gonadectomy, thyroid status and growth hormone deficiency) settings known to

be associated with energy homeostasis and alterations in insulin sensitivity. We have determined vaspin gene expression by real-time PCR. Vaspin was decreased after fasting and its levels were partially recovered after leptin treatment. Chronic treatment with metformin increased vaspin gene expression. Vaspin mRNA expression reached the highest peak at 45 days in both sexes after birth and its expression was higher in females than males, but its levels did not change throughout pregnancy. Finally, decreased levels of growth hormone and thyroid hormones suppressed vaspin expression. These findings suggest that WAT vaspin mRNA expression is regulated by nutritional status, and leptin seems to be the nutrient signal responsible for those changes. Vaspin is influenced by age and gender, and its expression is increased after treatment with insulin sensitizers. Finally, alterations in pituitary functions modify vaspin levels. Understanding the molecular mechanisms regulating vaspin will provide new insights into the pathogenesis of the metabolic syndrome.

Abbreviations

ACTH, adrenocorticotropic hormone; ACC, acetyl CoA carboxilase; CPT-1, carnitine palmitoyltransferase-1; FAM, 6-carboxy-fluorescein; FAS, fatty acid synthase; GLUT-1 and GLUT-4, glucose transporter-1 and 4; gWAT, gonadal white adipose tissue; GH, growth hormone; HPRT, hypoxanthineguanine phosphoribosyltransferase; ORX, orchidectomized; OLETF, Otsuka Long-Evans Tokushima Fatty; OVX, ovariectomized; RT-PCR, real-time reverse-transcription polymerase chain reaction; SDR, spontaneous dwarf rats; TAMRA, 6-carboxy-tetramethyl-rhodamine; Pb, Probe; TSH, thyroid-stimulating hormone; vaspin, visceral adipose tissue-derived serine protease inhibitor; WAT, white adipose tissue

The metabolic syndrome is a clustering of medical disorders associated with cardiovascular diseases, obesity, type 2 diabetes mellitus, hypertension and dyslipidaemia (Cornier *et al.* 2008). Therefore, therapeutic interventions that simultaneously address several risk factors in patients with the metabolic syndrome are needed to improve the multiple associated disorders. It is well established that the metabolic syndrome is closely associated with abdominal obesity (Despres *et al.* 2008). The WAT acts not only as a fatty acid depot, but also as an active endocrine organ that undergoes hyperplastic changes with relevant functions in obesity (Fruhbeck *et al.* 2001; Fruhbeck, 2008).

Currently, there are many studies being carried out on the adipocyte secreted insulin-sensitizing hormones 'adipokines', which play a central role in peripheral insulin resistance and the metabolic syndrome (Fruhbeck *et al.* 2001; Fruhbeck, 2008). During the last years, many of these adipokines have been identified and reported to exert relevant functions on energy and glucose homeostasis (Fruhbeck *et al.* 2001; Fernandez-Real *et al.* 2003; Fernandez-Real & Pickup, 2008). One of the latest adipokines is the visceral adipose tissue-derived serine protease inhibitor (vaspin). Vaspin is a member of the serine protease inhibitor family which has insulin-sensitizing effects (Hida *et al.* 2005). Vaspin was located in visceral WAT of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an animal model characterized by abdominal obesity with type 2 diabetes mellitus (Hida *et al.* 2005). Vaspin was specifically located in mature adipocytes isolated from different fat depots, whereas its expression was not found in stromal endothelial or vascular cells (Hida *et al.* 2005). Both circulating and adipocyte vaspin levels were significantly increased when the rats were obese and insulin resistant (Hida *et al.* 2005). Contrary to this, vaspin expression was decreased when rats developed diabetes and lost body weight, while vaspin serum levels were increased when diabetic rats were treated with pioglitazone and insulin (Hida *et al.* 2005). Therefore, it was suggested that vaspin may be a compensatory mechanism in response to decreased insulin sensitivity. Vaspin administration to obese mice improved glucose tolerance and insulin sensitivity and modified the expression

of genes involved in the pathogenesis of insulin resistance, such as resistin, leptin and adiponectin (Hida *et al.* 2005).

In clinical studies, the correlation between vaspin gene expression and serum levels of markers of insulin sensitivity and glucose metabolism is still unclear. Although vaspin was not detected in WAT from lean subjects (Kloting *et al.* 2006), it was located in both visceral and subcutaneous adipose tissue of obese patients (Kloting *et al.* 2006). Moreover, its levels were significantly correlated with obesity, fat mass, decreased insulin sensitivity and impaired glucose tolerance (Kloting *et al.* 2006). However, vaspin mRNA expression was only detectable in 23% of visceral and 15% of the subcutaneous adipose tissue samples (Kloting *et al.* 2006), and another report found that vaspin mRNA expression was predominantly located in non-fat cells (Fain *et al.* 2008). Vaspin serum concentrations were also associated with obesity and impaired insulin sensitivity (Youn *et al.* 2008). However, these correlations between circulating vaspin and the body mass index disappeared in patients with type 2 diabetes (Youn *et al.* 2008) or chronic haemodialysis (Seeger *et al.* 2008). Hence, much remains to be learned about the precise role of vaspin in glucose metabolism.

We therefore investigated the regulation of vaspin gene expression in rat WAT in different physiological (nutritional status, pregnancy, age and gender) and pathophysiological (gonadectomy, thyroid status and growth hormone deficiency) settings known to be associated with energy homeostasis and alterations in insulin sensitivity.

Methods

Animal models and strains

This study used different rat models: male and female Sprague–Dawley rats (bred in the animalario General USC; Santiago de Compostela, Spain); male dwarf (HsdOla: dw-4) Lewis rats (150–175 g, 10–12 weeks old) (Harlam Ibérica; Barcelona, Spain); and male Lewis rats age-matched with dwarf Lewis rats (250–300 g, 10–12 weeks old) (Harlam Ibérica). Rats were

housed under conditions of controlled illumination (12 h light–12 h dark cycle), humidity and temperature. Animals were fed with a standard diet and tap water *ad libitum* and eight animals per group were used in each experimental protocol. Surgical procedures were performed under anaesthesia by an intraperitoneal injection of ketamine–xylazine (ketamine, 100 mg (kg body weight)$^{-1}$, plus xylazine, 15 mg (kg body weight)$^{-1}$) as previously described (Caminos *et al.* 2008*a*). The animals were killed by decapitation in a room separate from other experimental animals in the afternoon (16.00–17.00 h) and gonadal fat (gWAT) pad was collected and frozen at –80°C until vaspin mRNA analysis. All experimental procedures in this study were reviewed and approved by the Ethics Committee of the University of Santiago de Compostela in accordance with our institutional guidelines and the European Union standards for the care and use of experimental animals.

Experimental designs

Experimental setting 1: Effects of food deprivation on vaspin mRNA expression This study examined the effects of food deprivation on gWAT vaspin mRNA expression in 10-week-old male rats. Groups of animals (n= 8 per group), were deprived of food for 24 and 48 h, while the control group was fed *ad libitum* (Nogueiras *et al.* 2003*a*, 2005). All animals had free access to tap water.

Experimental setting 2: Effects of peripheral leptin administration on vaspin mRNA expression The effects of systemic leptin administration on WAT vaspin mRNA expression were studied in 10-week-old male Sprague–Dawley rats as described elsewhere (Legradi *et al.* 1997) Rats were assigned to one of the following groups: the first group was allowed free access to food; the second group was food-deprived for 48 h; and the third group was fasted for 48 h and treated with recombinant leptin (Sigma) at a dose of 0.5 μg (kg body weight)$^{-1}$ every 6 h for 3 days (intraperitoneal injection).

Experimental setting 3: Effect of chronic metformin treatment on vaspin mRNA expression

To investigate whether the insulin-sensitizing agent metformin had any effect on WAT vaspin mRNA expression, four weight-matched groups of 10-week-old male Sprague–Dawley rats were treated with metformin (300 mg kg^{-1} day^{-1}) for 1, 2 and 3 weeks, or subcutaneous vehicle injection (control group), according to the method describe elsewhere (Nogueiras *et al.* 2004).

Experimental setting 4: Influence of age and gender on vaspin mRNA expression To analyse the effect of age and gender on WAT vaspin mRNA expression, male and female Sprague–Dawley rats were studied at the following ages: 15, 25, 45, 60 and 90 days old (Nogueiras *et al.* 2003*a*).

Experimental setting 5: Vaspin mRNA expression throughout gestation Vaspin mRNA expression was studied throughout gestation in WAT of Sprague–Dawley rats, according to the method described elsewhere (Nogueiras *et al.* 2003*b*; Caminos *et al.* 2008*b*). Female rats were mated on the day of proestrus at approximately 10 weeks old. The first day of pregnancy was documented by the presence of a vaginal plug with sperm after mating. gWAT was dissected from pregnant rats killed on days 12, 16, 19 and 21 of gestation.

Experimental setting 6: Effects of gonadal hormones on vaspin mRNA expression In order to analyse the effect of gonadal hormones on WAT vaspin mRNA expression 10-week-old male and female rats were bilaterally orchidectomized (ORX), ovariectomized (OVX) or sham operated as described previously (Nogueiras *et al.* 2005). Two weeks after surgery the different groups of rats were killed.

Experimental setting 7: Effect of thyroid status on vaspin mRNA expression The effect of thyroid status on WAT vaspin mRNA expression was evaluated as described elsewhere (Caminos *et al.* 2002, 2008*a*). Rats were rendered hypothyroid by 2 weeks of treatment with

0.1% amino-triazole (3-amino-1,2,4-triazole; Sigma, St Louis, MO, USA) in the drinking water and hyperthyroidism was induced in rats by chronic subcutaneous administration of l-thyroxine (100 mg day^{-1}l-thyroxine sodium salt pentahydrate (T4); Sigma) for 2 weeks. Establishment of altered thyroid status was confirmed as previously described (Caminos *et al.* 2008*a*). Aminotriazole treatment significantly increased plasma thyroid-stimulating hormone (TSH) levels whereas the administration of T4 significantly decreased plasma TSH levels (data not shown).

Experimental setting 8: Effect of growth hormone deficiency on vaspin mRNA expression To investigate the effects of growth hormone (GH) deficiency on WAT vaspin mRNA expression, spontaneous dwarf rats (SDR), a GH-deficient model and Lewis wild-type age-matched rats were used as described previously (Caminos *et al.* 2002).

RNA isolation and RT- PCR analysis

Vaspin mRNA expression was analysed by RT-PCR as previously validated (Caminos *et al.* 2005, 2008*a*; Lage *et al.* 2007). RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of total RNA were used for each RT reaction and cDNA synthesis was performed using the SuperScript First-Strand Synthesis System (Invitrogen) and random primers. Negative control reactions, containing all reagents except the sample, were used to ensure specificity of the PCR amplification. The resulting cDNA was subjected to PCR amplification using the LightCycler real-time PCR 2.0 (Roche Diagnostics, Germany) and software version 4.0 according to the method described elsewhere (Caminos *et al.* 2008*a*). The primer pairs were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 1). Data were normalized with respect to hypoxanthineguanine phosphoribosyltransferase (HPRT) level in each sample

(Caminos *et al.* 2008*a*). Following amplification, the specificity of the amplicon was confirmed by melting curve analysis.

For the analysis of gene expression of fatty acid synthase (FAS), acetyl CoA carboxilase (ACC), carnitine palmitoyltransferase-1 (CPT-1), adiponectin, glucose transporter-1 and 4 (GLUT-1 and GLUT-4) in the metformin treatment we use a different real-time PCR method: real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses were performed in a fluorescent temperature cycler (TaqMan; Applied Biosystems; Foster City, CA, USA) following the manufacturer's instructions (Vazquez *et al.* 2008; Gonzalez *et al.* 2008). Total RNA (500 ng) was used for each RT reaction of gWAT. The PCR cycling conditions included an initial denaturation at 50°C for 10 min followed by 40 cycles at 95°C for 15 s; and 60°C for 1 min. The oligonucleotide-specific primers and probes for rat FAS, ACC, CPT-1, adiponectin, GLUT-1, GLUT-4 and 18S are described in Table 1. For the analysis of the data, the input value of the gene expression was standardized to the 18S value for the sample group and was expressed compared with the average value for the control group.

Statistical analysis

The results are shown as the mean ±s.e.m. Significant differences ($P < 0.05$) between treated and control groups were determined using Student's unpaired *t* test, two tailed (GraphPad Instat, version 3.05).

Results

Influence of fasting and leptin on vaspin mRNA levels

After 24 and 48 h of food deprivation there was a significant ($P < 0.01$ and $P < 0.001$, respectively) decrease in WAT vaspin mRNA levels (Fig. 1A). The i.p. administration of leptin at a dose of 0.5 μg kg^{-1} every 6 h for 3 days failed to modify the body weight in fasted rats (Fig.

1B). However, leptin treatment partially reversed the low WAT vaspin mRNA expression observed after fasting ($P < 0.01$) (Fig. 1C), suggesting that the drop in vaspin mRNA expression after fasting might be mediated by serum leptin levels.

Influence of metformin on vaspin mRNA levels

Chronic subcutaneous treatment with metformin, an insulin sensitizer, for 3 weeks led to a significant decrease in body weight (Fig. 2A). Metformin administration increased ($P < 0.01$) expression of WAT vaspin mRNA when compared with vehicle-injected rats (Fig. 2B). The efficiency of the treatment was corroborated by the increased expression of the glucose transporters GLUT-1 and GLUT-4 (Fig. 2C), whereas no changes were found in the expression of some key enzymes involved in fatty acid synthesis (Fig. 2C).

Influence of age, gender and pregnancy on vaspin mRNA expression

The present study was performed to determine whether vaspin levels change throughout the development of the rat. The greatest level of expression was observed at 45 days ($P < 0.01$, 25 days *vs* 45 days) in both sexes, and it declined thereafter (45 days *vs* 60 days, $P < 0.001$; 45 days *vs* 90 days, $P < 0.01$). Vaspin mRNA levels were greater in 45-day-old female rats compared with their male counterparts of the same age, whereas its levels remained similar between males and females at other ages (Fig. 3). Taken together, these results suggest that WAT vaspin mRNA expression is specifically regulated by age and gender. Next, we assessed the influence of pregnancy, a physiological model of hyperleptinaemia and decreased insulin sensitivity, on the gWAT vaspin expression of pregnant rats. However, we failed to detect any change in vaspin mRNA levels throughout pregnancy (Fig. 4).

Influence of pituitary factors on vaspin mRNA levels

Hypophysectomized rats have been used as a model to study the effects of pituitary hormone deficiency, which results in a suppression of weight gain, a reduction in lean mass and decreased skeletal growth (Zapf, 1998). Hypophysectomized rats showed lower ($P < 0.01$) vaspin mRNA levels in comparison to control rats (Fig. 5A). The pituitary gland secretes several hormones that play a crucial role in the regulation of energy balance: (a) TSH that stimulates the thyroid gland; (b) follicle-stimulating hormone and luteinizing hormone that are involved in the synthesis of gonadal hormones; (c) growth hormone (GH) which, in addition to its role in linear growth, plays a major role in metabolic homeostasis; and (d) adrenocorticotropic hormone (ACTH) that acts on the cells of the adrenal cortex, stimulating them to produce glucocorticoids, mineralocorticoids and gonadal steroids. Therefore, we have assessed the specific effect of several pituitary hormones on vaspin expression. To further characterize the effect of gonadal hormones on vaspin expression, we gonadectomized prepubertal (21 days old) male and female rats until 45 days old when vaspin reached the highest peak of expression. We found that vaspin mRNA levels were unchanged in gonadectomized rats (Fig. 5B), indicating that gonadal hormones did not regulate WAT vaspin expression. Thyroid hormones play a crucial role in the regulation of growth, development and metabolism in vertebrates (Krotkiewski, 2002). As expected, hyperthyroid rats showed a clear suppression of body weight (Fig. 5C), whereas changes in hypothyroid rats were not so marked (Fig. 5C). No changes were found in glucose or insulin levels, but a clear decrease in serum triglycerides levels was detected in both hyperthyroid and hypothyroid rats (Table 2). Vaspin mRNA levels were significantly ($P < 0.01$) down-regulated in hyperthyroid rats and significantly ($P < 0.05$) increased in hypothyroid rats with respect to euthyroid rats (Fig. 5D). Therefore, our results indicated that WAT vaspin mRNA expression was affected by thyroid status. Finally, we investigated the effects of GH deficiency on vaspin. Dwarf rats showed lower body weight in comparison with control rats (Fig. 5E). GH-deficient rats had lower insulin levels, but no differences were

observed in glucose or triglycerides levels (Table 3). We found that WAT vaspin mRNA expression was significantly ($P < 0.05$) down-regulated in GH-deficient rats compared with age-matched control rats (Fig. 5F), suggesting that the lack of GH decreased vaspin levels.

Discussion

Obesity is a heterogeneous condition in which body fat distribution largely determines metabolic perturbations. Consequently, individuals characterized by increased visceral fat deposition have a higher risk of developing insulin resistance, type 2 diabetes and coronary artery disease (Despres *et al.* 2008). Although the biological mechanisms underlying the adverse impact of visceral fat accumulation remain to be established, it seems clear that adipokines secreted by visceral adipose tissue might be of clinical relevance for treating the metabolic syndrome. Vaspin is a recently discovered visceral adipose tissue-derived factor that exhibits insulin-sensitizing effects (Hida *et al.* 2005), since its administration to obese rodents ameliorated certain obesity-associated disorders (Hida *et al.* 2005). However, it is unknown whether vaspin is causative or protective in the development of obesity and metabolic disorders (Li *et al.* 2008).

In the work presented here, we have investigated the influence of different physiological and pathological conditions known to be involved in the regulation of energy balance and/or glucose metabolism on vaspin gene expression. It was reported that vaspin mRNA expression was increased when OLEFT rats were obese and insulin resistant, and its levels were decreased when the same animals developed a severe hyperglycaemia and lost body weight (Hida *et al.* 2005). Thereby, we evaluated the effect of total caloric restriction, and we found a marked decrease in WAT vaspin mRNA expression after fasting. Since fasting is a hypoleptinaemic state, we challenged fasted rats with leptin and found that leptin administration was able to partially reverse the lower WAT vaspin levels after fasting. Taken together, our current findings indicate

that vaspin is strongly regulated by nutritional status and leptin is one of the nutritional signals modulating those changes.

Pregnancy is characterized by a series of metabolic changes which promote adipose tissue accretion in early gestation in order to meet increased metabolic demands. During pregnancy there is a marked increase in food intake, as a consequence of changes in the set-point of several orexigenic and anorexigenic signals. In addition, during gestation there are clear changes in several mechanisms related to glucose homeostasis (Armitage *et al.* 2008), due to the decrease in insulin sensitivity by peripheral tissues, which leads to higher demands of insulin during gestation. In fact, gestational diabetes is the most common metabolic abnormality during pregnancy. Here, we failed to detect any change in vaspin gene expression throughout pregnancy. Overall, our data suggest that the increased circulating leptin levels in gestation do not modulate vaspin expression, suggesting that vaspin is not an important factor for the changes observed in glucose metabolism during this state.

It was reported that vaspin gene expression was decreased in diabetic rats, while the treatment with insulin and thiazolidinedione induced vaspin gene expression (Hida *et al.* 2005), suggesting that vaspin may be a compensatory mechanism in response to decreased insulin sensitivity or impairment in glucose metabolism (Hida *et al.* 2005). In the present study, we investigated the effects of metformin, which enhances peripheral glucose uptake and improves insulin sensitivity (Edgerton *et al.* 2009). Our results demonstrated that a long-term treatment with metformin increased WAT vaspin mRNA expression. Therefore, our findings are in agreement with previous reports showing that the treatment with thiazolidinedione, an insulin sensitizer, induced vaspin gene expression (Hida *et al.* 2005). Overall, the results suggest that enhanced insulin sensitivity in rodents increases vaspin levels. Intriguingly, data obtained in humans showed that patients on metformin therapy had lower serum vaspin levels than patients who were not taking metformin (Gulcelik *et al.* 2009), suggesting that vaspin might be differentially regulated in humans and rodents.

There is a wealth of clinical and experimental data demonstrating that sex steroids and insulin interact in their effects on several tissues (Livingstone & Collison, 2002). At physiological levels, testosterone and oestrogens are thought to be involved in maintaining normal insulin sensitivity (Ropero *et al.* 2008). For example, high circulating levels of sex steroids appear to contribute to the development of insulin resistance, and women with low serum oestrogens are at greater risk of developing type 2 diabetes (Ropero *et al.* 2008). While it is clear that the serum levels of sex steroids are closely linked to insulin sensitivity, the nature of this link is still unclear. In this paper we showed that vaspin mRNA levels were age dependent. While they were low in 25-day-old male and female rats, they markedly increased during puberty, reaching the highest levels at 45 days in both sexes and declining thereafter. This is particularly interesting since the elevated serum levels of sex steroids associated with normal puberty are associated with a decrease in insulin sensitivity (Ropero *et al.* 2008). To test the hypothesis that increased vaspin levels might be involved in the insulin resistance present in these animal subjects, we carried out additional experiments in gonadectomized rats. It has been previously shown that in the absence of endogenous production of sex steroids, insulin sensitivity is reduced (Pasquali *et al.* 2002; Ding *et al.* 2006). Contrary to our expectations, we found that WAT vaspin mRNA levels were unchanged in gonadectomized rats. Thus, it appears unlikely that the insulin resistance present in pubertal animals is mediated by vaspin. These data are also in agreement with those reported here in pregnant rats where, despite marked changes in female gonadal function, no change in vaspin mRNA was seen. Furthermore, our data are in agreement with clinical studies showing that serum concentrations of vaspin are significantly higher in female compared with male subjects (Youn *et al.* 2008; Seeger *et al.* 2008).

It is well known that alterations in thyroid function of GH are associated in the clinical setting with marked changes in body weight, glucose homeostasis and insulin sensitivity (Pasquali *et al.* 2002). Thyroid dysfunction is frequently associated with metabolic changes that affect fat mass, adipocyte function (Pontikides & Krassas, 2007) and secretion of several adipokines (Iglesias & Diez, 2007). Our results demonstrated that WAT vaspin mRNA levels were lower

in hyperthyroid rats and higher in hypothyroid rats in comparison to euthyroid animals. Therefore, our findings suggest that thyroid status plays an important role in the regulation of vaspin. GH is known to have deep effects on adipose tissue and body composition (Perrini *et al.* 2008). GH deficiency is usually associated with increased adiposity and impairment of insulin sensitivity, whereas GH excess is normally related with leanness, insulin resistance and hyperinsulinaemia in both humans and animals (Davies *et al.* 2007; Park *et al.* 2008). Our data showed that WAT vaspin mRNA levels are lower in GH-deficient rats compared to control rats, suggesting that vaspin may be particularly sensitive to the influence of GH. Overall, our results demonstrated that a failure in the thyroid neuroendocrine axis, as well as in the GH system, decreased the expression of WAT vaspin mRNA, suggesting that somatotroph cell function plays a critical role on vaspin regulation.

In summary, our data indicate that (a) WAT vaspin mRNA expression is regulated by nutritional status, and leptin is one of the nutritional signals responsible for those changes, (b) vaspin is influenced by age and gender, (c) vaspin is increased after treatment with insulin sensitizers, and (d) alterations in pituitary functions modify vaspin levels. Further studies will be necessary for a better understanding of the molecular mechanisms regulating vaspin, providing new insights into the pathogenesis of the metabolic syndrome.

Author contributions

C.R.G, J.E.C, M.J.V., G.R.M.E, S.S.A, S.B., M.F.G., L.A.C., A.C.G, A.A. conception, analysis and interpretation of data; J.E.C, R.N. and C.R.G. drafting the article; R.N., M.L., C.D. revising it critically for important intellectual content, and final approval of the version to be published. The experiments were done in the Department of Physiology, Faculty of Medicine, University of Santiago de Compostela (Spain).

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Table 1. Primers used for RT-PCR and real time PCR analysis

Gene	Forward and reverse primer	Gen-Bank accession No.
Rat vaspin	Forward: 5′-AGTCGGAAAACCCACAACAG -3′ Reverse: 5′-CGGTCTTGCCTTCCTCTATG -3′	AF245398
Rat HPRT	Forward: 5′-CAGTCCCAGCGTCGTGATTA-3′ Reverse: 5′-AGCAAGTCTTTCAGTCCTGTC-3′	NM_013556
Rat GLUT-1 (Slc2a1)	Forward: 5′-GCATCGTCGTTGGGATCCT-3′ Reverse: 5′-CAAGTCTGCATTGCCCATGA-3′ Pb: FAM-5′-TTGCCCAGGTGTTCGGCTTAGACTCC-3′-TAMRA	NM_138827.1
Rat GLUT-4 (Slc2a4)	Forward: 5′-CATTCTCGGACGGTTCCTCAT-3′ Reverse: 5′-GCGATTTCTCCCACATACATAGG-3′ Pb: FAM-5′-CGCCTACTCAGGGCTAACATCAGGGTTG-3′-TAMRA	NM_012751.1
Rat 18S	FW: 5′-CGGCTACCACATCCAAGGAA-3′ RV: 5′ -GCTGGAATTACCGCGGCT-3′ Pb: FAM-5′-GACGGCAAGTCTGGTGCCAGCA-3′-TAMRA	M11188
Rat AdipoQ	Rn00595250_m1(Gene Expression Assay Applied Biosystems)	NM_144744.2
Rat ACCa (Acaca)	Forward: 5′-TGGGCGGGATGGTCTCTTT-3′ Reverse: 5′-AGTCGCAGAAGCCCATT-3′ Pb: FAM-5′-ACCTTTGAAGATTTCGTCAGGATCTTTGATGA-3′-TAMRA	NM_022193.1
Rat FAS (Fasn)	Forward: 5′-GAC ATTTCATCAGGCCACC-3′ Reverse: 5′ -CCTCTAGCAGCCGCACCTC-3′ Pb: FAM-5′-CTGCCCAGGACAGGAACCG-3′-TAMRA	NM_017332.1
Rat CPT-1b	Rn01407782_g1(Gene Expression Assay Applied Biosystems)	NM_013200.1

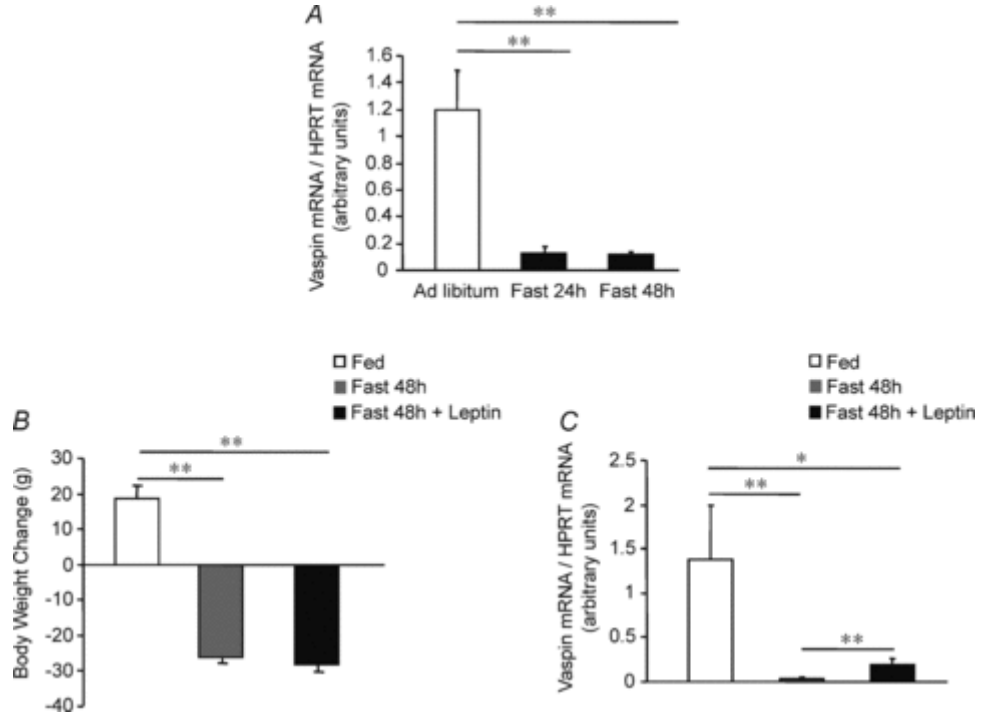

Figure 1. Effects of nutritional status on vaspin mRNA expression in gWAT of male rats
A, 24 h and 48 h fasting caused a significant ($P < 0.01$) decrease on vaspin mRNA levels. *B*, body weight change in rats after 48 h fasting and in fasted rats treated with leptin. *C*, leptin treatment reversed the low levels of vaspin mRNA after 48 h fasting. Values were normalized to those of the internal control hypoxanthine phosphoribosyl transferase (HPRT), and the results are expressed as arbitrary units. Mean values were obtained from 8 animals per group. Values are the mean ±s.e.m., *$P < 0.05$, **$P < 0.01$.

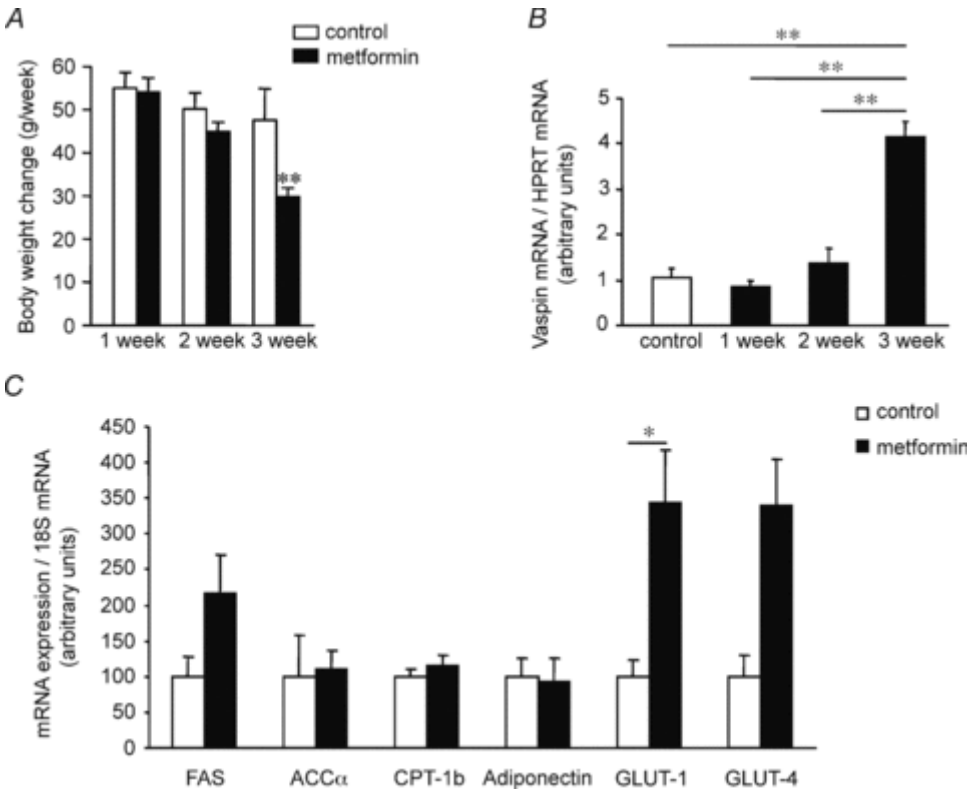

Figure 2. Analysis of vaspin mRNA expression in gWAT after metformin administration
A, metformin treatment decreased body weight after 3 weeks. *B*, metformin treatment for 3 weeks enhanced gWAT vaspin mRNA expression after 3 weeks. *C*, metformin treatment for 3 weeks increased the expression of GLUT-1 and GLUT-4 in gWAT, but not adiponectin, FAS, ACCα or CPT-1b. Values were normalized to those of the internal control HPRT and 18S. The results are expressed as arbitrary units. Mean values were obtained from 8 animals per group. Values are the mean ±s.e.m., **P* < 0.05, ***P* < 0.01.

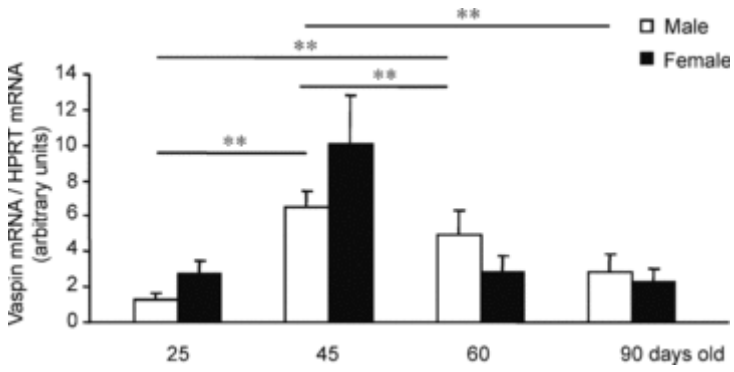

Figure 3. Analysis of gWAT vaspin mRNA expression from 25-, 45-, 60- and 90-day-old male and female rats
Vaspin mRNA significantly increased after 45 days in both female and male rats. Values were normalized to those of the internal control HPRT, and the results are expressed as arbitrary units. Mean values were obtained from 8 animals per group. Values are the mean ±s.e.m., $^{**}P < 0.01$.

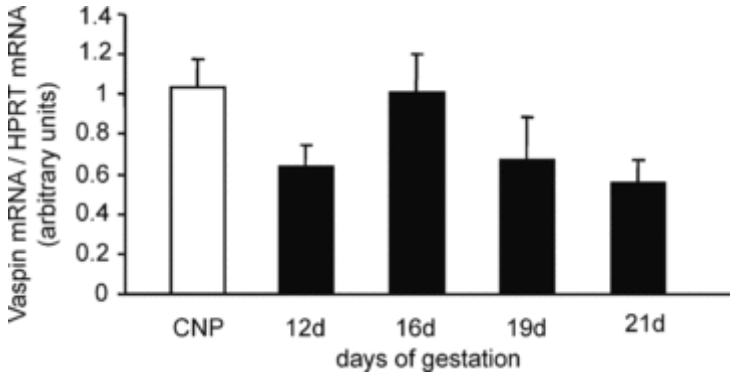

Figure 4. Vaspin mRNA expression throughout gestation in gWAT
Values were normalized to those of the internal control HPRT, and the results are expressed as arbitrary units. Mean values were obtained from 8 animals per group. Values are the mean ±s.e.m.

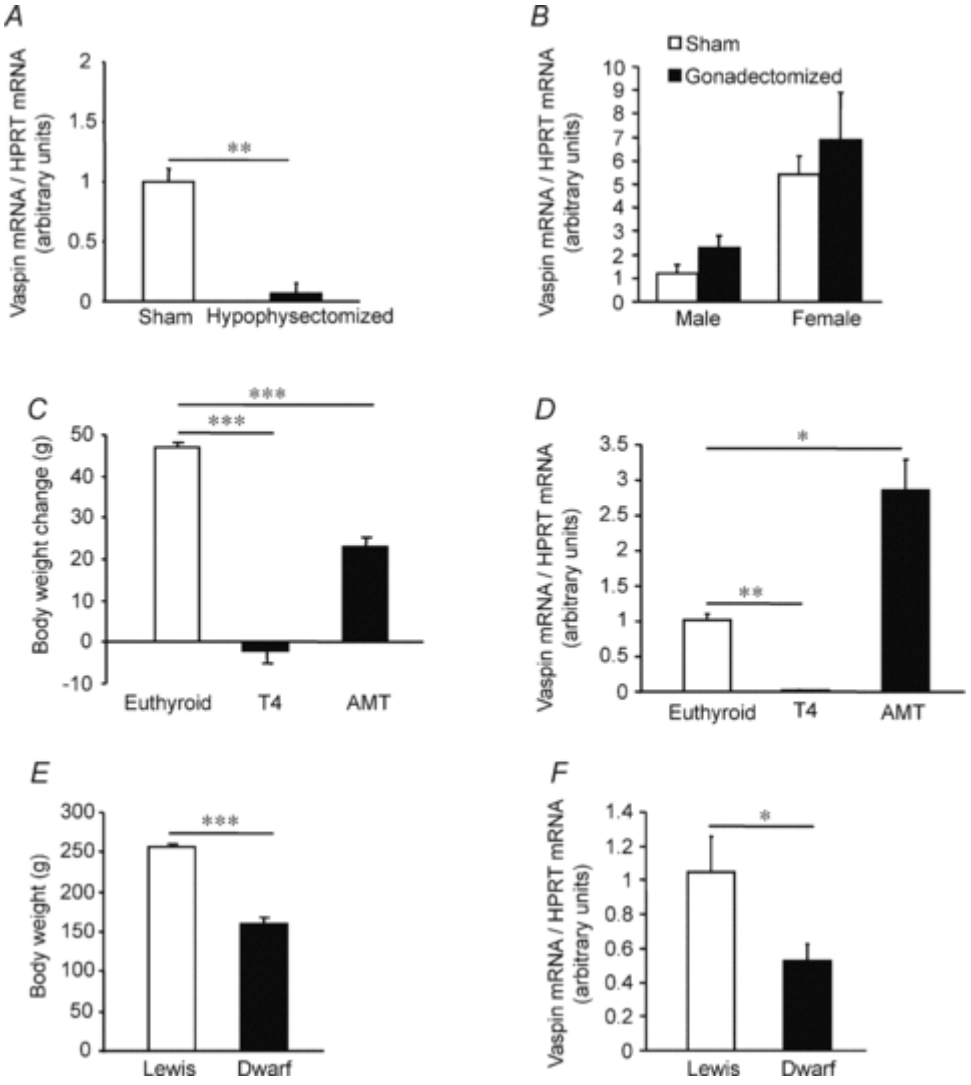

Figure 5. Analysis of pituitary factors on gWAT vaspin mRNA expression
A, hypophysectomy decreased vaspin mRNA expression. *B*, gonadectomy did not modify gWAT vaspin levels in males or females. *C*, hyperthyroid and hypothyroid rats showed a decreased body weight when compared to euthyroid rats. *D*, vaspin mRNA levels were significantly down-regulated in response to hyperthyroidism and increased hypothyroidism when compared with the euthyroid group rats. *E*, dwarf rats showed a decreased body weight when compared to control rats. *F*, vaspin mRNA expression was significantly down-regulated in dwarf rats compared with age-matched control Lewis rats. Values were normalized to those of the internal control HPRT, and the results are expressed as arbitrary units. Mean values were obtained from 8 animals per group. AMT, amino-triazol; T4, l−thyroxine. Values are the mean ±s.e.m., *$P < 0.05$, **$P < 0.01$.

Table 2. Insulin, glucose and trygliceride levels in euthyroid, hyperthyroid and hypothyroid rats

Treatment	Condition	Value
Insulin (ng ml^{-1})	Euthyroid	2.10 ± 0.34
Hyperthyroid (T4)	2.48 ± 0.4	Hypothyroid (AMT)
1.50 ± 0.54	Glucose (nmol l^{-1})	Euthyroid
7.36 ± 0.14	Hyperthyroid (T4)	7.38 ± 0.35
Hypothyroid (AMT)	6.12 ± 0.22	TGs (mg dl^{-1})
Euthyroid	142.55 ± 16.5	Hyperthyroid (T4)
81.04 ± 7.44 **	Hypothyroid (AMT)	38.65 ± 4.6 ***

Table 3. Insulin, glucose and trygliceride levels in wild-type Lewis and Dwarf rats. ***p < 0.001

Treatment	Rat strain	Value
Insulin (ng ml^{-1})	Wild-type Lewis	1.38 ± 0.23
	Dwarf	0.65 ± 0.09 ***
Glucose (mg dl^{-1})	Wild-type Lewis	79.71 ± 2.83
	Dwarf	86.63 ± 3.95
TGs (mg dl^{-1})	Wild-type Lewis	108.57 ± 12.63
	Dwarf	117.39 ± 19.95