The Matricellular Protein SPARC/Osteonectin as a Newly Identified Factor Up-regulated in Obesity

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Alterations in the expression level of genes may contribute to the development and pathophysiology of obesity. To find genes differentially expressed in adipose tissue during obesity, we performed suppression subtractive hybridization on epididymal fat mRNA from goldthioglucose (GTG) obese mice and from their lean littermates. We identified the secreted protein acidic and rich in cysteine (SPARC), a protein that mediates cell-matrix interactions and plays a role in modulation of cell adhesion, differentiation, and angiogenesis. SPARC mRNA expression in adipose tissue was markedly increased (between 3- and 6-fold) in three different models of obesity, i.e. GTG mice, ob/ob mice, and AKR mice, after 6 weeks of a high fat diet. Immunoblotting of adipocyte extracts revealed a similar increase in protein level. Using a SPARC-specific ELISA, we demonstrated that SPARC is secreted by isolated adipocytes. We found that insulin administration to mice increased SPARC mRNA levels in the adipose tissue. Food deprivation had no effect on SPARC expression, but after high fat refeeding SPARC mRNA levels were significantly increased. Our results reveal both hormonal and nutritional regulation of SPARC expression in the adipocyte, and importantly, its alteration in obesity. Finally, we show that purified SPARC increased mRNA levels of plasminogen activator inhibitor 1 (PAI-1) in cultured rat adipose tissue suggesting that elevated adipocyte expression of SPARC might contribute to the abnormal expression of PAI-1 observed in obesity. We propose that SPARC is a newly identified autocrine/paracrine factor that could affect key functions in adipose tissue physiology and pathology.

Obesity results from a chronic imbalance between energy intake and energy expenditure. This syndrome is commonly associated with increased risks of cardiovascular problems and metabolic abnormalities, including hypertension, hyperinsulinemia, insulin resistance, and type 2 diabetes (1, 2). Obesity is defined as a pathological excess of fat mass. Adipose tissue grows throughout life by increasing the size and volume of preexisting adipocytes and de novo recruitment of preadipocytes. Adipose tissue is the major site for energy storage and disposal and is also implicated in glucose homeostasis. Today, it is well recognized that adipocytes play a central role in metabolism through the secretion of signaling factors that regulate food intake and metabolic efficiency (3, 4).

Recent research on obesity has revealed that body weight regulation is a complex phenomenon that is partly genetically defined. Body weight is determined by multiple interactions between genes and environmental factors such as dietary composition, total caloric intake, and exercise (1, 5). Multiple genes appear to contribute to the pathogenesis of obesity (6). Several studies have identified the existence of monogenic obesity in mice and, more recently, cases of monogenic obesity in humans have been reported. However, the mutations identified so far explain only a minute proportion of human obesity syndromes (6). Despite intensive efforts, the nature of the molecular defects leading to the occurrence and progression of the common obesity is still poorly understood.

It is generally accepted that alterations in gene expression may contribute to the pathogenesis and physiopathology of this syndrome. Numerous studies have focused on the expression of specific candidate genes at the RNA level (7–9). For example, it has been shown that obesity is associated with elevated expression of plasminogen activator inhibitor 1 (PAI-1)1 or tumor necrosis factor α (TNF-α) in the adipose tissue (10, 11). Interestingly, these defects have been associated with the insulin-resistant state and cardiovascular risk, two obesity-related complications (12, 13). At present, a limited number of studies have screened for new genes of which the expression is modulated by obesity and thus might contribute to the obese phenotype (14–18).

One attractive strategy for finding obesity-related genes involves examining differential gene expression in adipose tissue of obese animal models. Here we employed the power of the SSH method (19) to identify mRNA that are differentially expressed in the adipose tissue of non-obese mice compared with obese ones. We used the GTG obese mice, which is a well studied model of obesity (20). In these mice, obesity is obtained after the injection of GTG, which destroys part of the hypothalamus and thus induces hyperphagia with ensuing obesity, hyperinsulinemia, and insulin resistance. With this approach, we found that the expression of SPARC (also known as osteonectin

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1 The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; SPARC, secreted protein acidic and rich in cysteine; TGF-β1, transforming growth factor-β1; GTG, goldthioglucose; SSH, suppression subtractive hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; ELISA, enzyme-linked immunosorbent assay.
or BM-40) is strongly elevated in the adipocyte of obese mice. We studied also the modulation of SPARC expression in physiologic and pathophysiologic states of the adipose tissue in rodents. Finally, we demonstrated that SPARC might be implicated in the regulation of PAI-1 mRNA expression in the adipose tissue.

MATERIALS AND METHODS

Animals and Experimental Design—Male OF1 mice were from Iffa-Credo (L’Arbresle, France). Male AKR/OlaHsd mice, male C57BL/6Stbr1/Br mice and their lean controls C57BL/6J mice were obtained at 4–10 weeks of age from Harlan France (Gannat, France). Wistar rats (150–200 g in body weight) were obtained from Iffa-Credo. Four animals were kept per cage in a temperature-controlled room (22 °C) with a 12-hour light-dark cycle. Water and food (standard laboratory chow diet from UAR, Epinay-S/Orge, France) were available ad libitum, except when indicated. GTG obese mice were generated by double injection of goldthioglucose (Sigma, St Louis, MO) administered to 3-week-old OF1 mice as previously described (20). Matched lean littermate controls were studied in parallel. The development of high fat diet-induced obesity was studied in the AKR/OlaHsd mice (21). At 5 weeks of age, mice were randomly assigned to receive either a chow diet containing 12% of calories from fat or a Western high fat diet (Teklad Adlipo 8812; Harlan Teklad, Madison, WI). Mice were pair-fed with a diet containing 42% of calories from fat. For 10 weeks the mice were weighed twice a week. After 3, 6, and 10 weeks on these diets animals were euthanatized for1h at 90 °C, and the protein concentration was assayed by bichoninic acid technique (Pierce). Proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot using a monoclonal antibody to SPARC (Hematologic Technologies, Essex Junction, VT). Immunoreactive proteins were detected by enhanced chemiluminescence. SPARC levels in the conditioned medium of rat adipocytes were measured with the ELISA kit from Hematologic Technologies according to the manufacturer's instructions. After cell fractionation, 200 µl of adipocytes were resuspended in 2 ml of Dulbecco's modified Eagle's medium supplemented with 0.5% (w/v) bovine serum albumin and 25 mm Hepes, pH 7.4. The adipocyte samples were left unstimulated or stimulated with 200 µg/ml of human purified osteonectin/SPARC (Calbiochem-Novabiochem Corp.) or 10 ng/ml of recombinant human transforming growth factor-β1 (TGF-β1). The plates were incubated at 37 °C under 5% CO₂ for 6 h. The samples were collected, total RNA was isolated, and were analyzed by Northern blot.

RESULTS

SSH Reveals SPARC as a Newly Identified Gene Differentially Expressed in GTG and ob/ob Mice—The purpose of our work was to reveal genes differentially expressed during obesity. We used SSH to detect changes in mRNA expression in white adipose tissue of obese GTG mice and their lean controls. cDNAs were prepared by reverse transcription and subjected to subtractive hybridization as previously described (Ref. 19; and “Materials and Methods”). A minilibrary was generated by randomly subcloning the subtracted PCR products. Over two hundred cDNA plasmids from the library were then subjected to differential screening using lean and obese subtracted cDNAs as probes. Subsequent analysis on Northern blots identified and confirmed six gene products differentially expressed in adipose tissue of GTG obese mice (data not shown). Primary DNA analysis of one of those clones revealed a sequence corresponding to that present in the 3'-untranslated region of the mouse SPARC gene (25). SPARC is a non-structural component of the extracellular matrix that modulates cell-matrix interaction (26, 27). During embryogenesis its expression is time- and tissue-specific. In the adult, SPARC expression has been associated with wound repair, tumorigenesis, and cataractogenesis (28, 29). Because of its known functional roles and pattern of expression, SPARC was chosen for further characterization. Northern blot hybridization with a cDNA probe encoding the full-length SPARC protein confirmed increased steady-state levels of the major SPARC transcript (2.2 kilobases) in adipose tissue of obese GTG mice. Adipose tissue of obese mice showed approximately a 5- to 6-fold increase in the

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expression of the main SPARC message (Fig. 1A, lanes 1 and 2). An increased level of SPARC mRNA was also observed in the subcutaneous adipose tissue of obese mice (data not shown). SPARC expression was also examined in liver and skeletal muscle, two other insulin-responsive tissues implicated in glucose homeostasis. No expression was observed in the liver (Fig. 1A, lanes 3 and 4). In contrast, SPARC messages were detected in skeletal muscle but levels were lower than in white fat. However, the amount of SPARC mRNA expression in this tissue was no different between lean and obese mice (Fig. 1A, lanes 5 and 6).

To test the hypothesis that obesity might be associated to abnormal expression of SPARC in adipose tissue, we looked at SPARC mRNA levels in the ob/ob mice and their lean littermates (+/−). In the genetic ob/ob model, obesity results from the disruption of the leptin signaling system (30). The ob/ob mouse lacks functional leptin and as a consequence is associated with hyperphagia, massive obesity, hyperinsulinemia, and severe insulin resistance (31). As shown in Fig. 1B, in ob/ob mice the two SPARC transcripts were induced ~3-fold compared with lean counterparts (lanes 1 and 2). This demonstrates that up-regulation of SPARC mRNA expression in obese white fat does not depend on a functional leptin signaling system. Besides adipocytes, fat tissue contains several other cell types, including preadipocytes, endothelial cells, smooth muscle cells, fibroblasts, mast cells, and macrophages. To determine the source of SPARC expression in adipose tissue, fat pads from lean (+/−) and ob/ob mice were digested with collagenase and then subjected to differential centrifugation to separate mature fat cells from non-adipose cells. The amount of SPARC mRNA associated with the adipocytes fraction was shown as a positive marker of the adipocyte fraction. Fig. 1C shows that the majority of SPARC mRNA was fractionated with the adipocytes (lanes 1–4) and as expected, SPARC expression was increased in adipocytes of obese mice (lanes 3 and 4). SPARC mRNA was also detected, but to a lesser degree in the stromal vascular fraction (lanes 5 and 6). Because of the relatively strong signal in the adipocyte fraction as compared with the stromal vascular fraction, we conclude that SPARC mRNA expression and its increase in the adipose tissue of obese mice are mainly found in adipocytes. In situ hybridization is needed to localize more precisely SPARC mRNA in the different adipose tissue components.

Expression of SPARC mRNA during Development of High Fat Diet-induced Obesity—To gain further insight into the regulation of SPARC in obesity, we examined SPARC expression in a model of diet-induced obesity. We studied the development of obesity in the obesity-prone AKR strain in response to high fat diet (21). AKR mice were divided into a normal diet group and a high fat diet group, and assigned to receive the two diets for up to 10 weeks. Fig. 2A shows the body weight gain within

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Fig. 1. Expression of SPARC mRNA in lean and obese mice. A, Northern blot analysis was performed using 15 μg of total RNA isolated from white adipose tissue (WAT), liver, and skeletal muscle of lean or GTG animals (n = 4 for each group). Levels of SPARC mRNA (indicated by arrows) were analyzed using a 32P-labeled SPARC cDNA probe. GAPDH mRNA is shown as a control for the loading and integrity of RNA. B, Northern blot analysis of SPARC mRNA levels (15 μg of RNA/lane) in white adipose tissue of 4-month-old ob/ob mice (n = 4) and of their lean counterparts (+/−) (n = 8). GAPDH mRNA is shown as a control for the loading and integrity of RNA. C, adipocytes and stromal vascular fraction from lean (+/−) or ob/ob mice were separated by collagenase digestion followed by centrifugation. 15 μg of total RNA isolated from the whole fat tissue or from each cellular fraction was analyzed for expression of SPARC by Northern blot. The blot was subsequently probed with aP2 and GAPDH as controls.
the two groups of mice. As previously described, on a high fat diet AKR mice developed a marked obesity compared with mice fed on a standard chow (21). At the end of the study, fat pads from fat fed animals weighed 4-fold more than those of control animals (1.9 \( \pm \) 0.2 versus 0.4 \( \pm \) 0.05; Fig. 2B). Epididymal fat pad weights of AKR mice maintained on chow or on high fat diet. Values are mean \( \pm \) S.E. (n = 6 per group). C and E, representative Northern blots.

Expression of SPARC mRNA during development of high fat diet-induced obesity in AKR mice. Initially, the obesity-prone AKR strain was fed a standard chow diet. At 5 weeks of age, half of the mice were switched to a high fat diet. After 3, 6, and 10 weeks on these diets, animals (n = 6 per group) were euthanatized and the epididymal fat depots were removed and weighed. Total RNA was prepared and examined for the expression of SPARC by Northern blot hybridization. The blots were subsequently probed with GAPDH as a control. A, growth curves of high fat and chow fed animals. B and D, epididymal fat pad weights of AKR mice maintained on chow or on high fat diet. Values are mean \( \pm \) S.E. (n = 6 per group). C and E, representative Northern blots.

Fig. 2. Expression of SPARC mRNA during development of high fat diet-induced obesity in AKR mice.

Expression and Secretion of SPARC by the Adipocyte—Next we confirmed by immunoblotting that the protein SPARC was also increased in the adipocytes of obese mice (Fig. 3A). SPARC expression was between 3- and 4-fold higher in protein extracts of ob/ob mouse adipocytes as compared with that of lean adipocytes. Because SPARC is a secreted molecule, we examined the ability of adipocytes to secrete SPARC ex vivo. The concentration of SPARC antigen in 16 h-conditioned medium of freshly isolated rat adipocytes was measured by a SPARC-specific ELISA (Fig. 3B). A \( \approx \) 7-fold higher amount of SPARC was observed in adipocyte-conditioned medium compared with control medium. This suggests that in our experimental system mature fat cells produce and secrete SPARC. We also found that the secretion of SPARC by adipocytes isolated from ob/ob mice was higher than that of adipocytes from lean mice (data not shown).

Expression of the SPARC-related Gene SC1 in Adipose Tissue of Obese Mice—As elevated expression of SPARC in adipocytes...
is likely to be associated with the increase in adipose mass that occurs in obesity, we investigated whether the expression of SC1 (also known as hevin), a SPARC-related protein, is also modulated in the adipose tissue of obese mice. SPARC and SC1 are members of a small family of proteins that are defined by a conserved carboxyl-terminal domain, followed by two conserved domains, a follistatin-like and an extracellular calcium-binding domain (32). SC1 shows the highest similarity with SPARC (70% identity at the amino acid level) and has been proposed to functionally compensate for the loss of SPARC in SPARC-null mice (33). RT-PCR studies of SC1 message level in white adipose tissue from 20-week-old obese GGT and ob/ob mice compared with that of lean counterparts are presented in Fig. 4. SC1 mRNA expression was upregulated in the white adipose tissue of the two lean strains (lanes 1 and 3). However, contrary to SPARC, SC1 mRNA expression was not affected by obesity at least at the time point studied (lanes 1 and 3 versus lanes 2 and 4, respectively). This suggests that altered adipogenic expression of SPARC could have specific functional consequences in obesity.

**Effects of Insulin Administration and Food Intake on SPARC mRNA Expression**—Because obesity in both humans and rodents is often associated with hyperinsulinemia (1, 2), we hypothesized that insulin could have an effect on the elevation of SPARC expression by obesity. We examined SPARC expression in the adipose tissue of fasted control mice after insulin administration. RNA was extracted from the fat pads at 2 and 4 h after injection of insulin. SPARC expression was examined by Northern blot (Fig. 5A). As a positive control for adipocyte genes induced by insulin, we analyzed the expression of ob gene coding for leptin (34). Insulin induced a time-dependent increase in both SPARC and ob messages. More precisely, mice injected with insulin for 4 h showed a 3.3-fold increase in the expression of SPARC mRNA in the adipose tissue (lanes 1 and 3). This suggests that insulin could have a key role as a specific stimulus of SPARC gene expression in adipose cells. However, the insulin effect on SPARC expression could be because of changes in glycemia rather than to a direct effect of insulin. Indeed, insulin induces a hypoglycemic response that can be associated with counter-regulatory hormonal mechanisms. Further studies are needed to more precisely define the regulatory role of insulin on SPARC expression. Because food intake increases plasma insulin concentration, we also examined the effect of fasting and refeeding on SPARC gene expression. Mice were first divided into a fed control group and a fasted one. After an overnight fast, the second group of animals was then divided into three groups; one group that served as a fasting control and two other groups that were re-fed with standard diet or with high fat diet for 6 h. SPARC and leptin mRNA expression in fat pads were analyzed by Northern blot (Fig. 5B). Food deprivation for 24 h did not markedly affect SPARC mRNA levels (lanes 1 and 2). Whereas refeeding mice with the high fat diet induced a modest increase in the level of SPARC messages (lane 4), high fat refeeding doubled SPARC levels compared with fasted controls (lanes 3 and 5). As previously shown (34), under fasting conditions, ob mRNA amount was decreased to barely detectable levels (lanes 1 and 2) but was restored to normal within 6 h after refeeding with both diets (lanes 4 and 5). Because it is widely accepted that a high fat diet tends to cause obesity, our findings suggest that consumption of a diet rich in fat could participate in the elevation of SPARC expression in the adipose tissue of mice.

**SPARC Modulates PAI-1 mRNA Expression in Adipose Tissue**—Finally, we approached the issue of SPARC action on the adipose tissue. SPARC has been shown to modulate the expression of a subset of extracellular matrix proteins implicated in angiogenesis (35). SPARC also stimulates PAI-1 biosynthesis by bovine endothelial cells (36). Because increased expression of PAI-1 by the adipose tissue is associated with obesity (11, 37, 38), we looked at the effect of SPARC on PAI-1 gene expression in cultured rat adipose tissue. At the same time we studied the effect of TGF-β1, which has been shown to induce PAI-1 expression in this tissue (38, 39). Samples of whole adipose tissue were exposed to purified SPARC or TGF-β1 for 6 h, and PAI-1 mRNA expression was analyzed by Northern blot (Fig. 6). Addition of SPARC led to a 1.8-fold increase in PAI-1 mRNA accumulation after correction for variability in the 18 S rRNA.
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Fig. 6. Effect of SPARC on PAI-1 mRNA expression in cultured adipose tissue. Rat adipose tissue was cultured in medium alone (control) or medium containing 20 μg/ml SPARC or 10 ng/ml TGF-β1 for 6 h. 15 μg of total RNA isolated from each sample was analyzed for PAI-1 mRNA expression by Northern blot. RNA was hybridized with an 18 S rRNA probe as a control. Fold increase was determined after densitometric scanning of the representative autoradiogram. Values (lane 2, × 1.8; lane 3, × 1.9) were obtained after normalization to the signal obtained with the 18 S rRNA probe.

signal (lane 2), and this increment was comparable with that obtained with TGF-β1 (lane 3). Our observation demonstrates that the adipose tissue responds to exogenous SPARC and that SPARC may function as a novel signaling molecule for this tissue.

DISCUSSION

We used the SSH strategy to isolate candidate genes that are dysregulated in the adipose tissue of obese mice. One of the genes revealed codes for SPARC, a matricellular protein that it thought to modulate cell-matrix interaction (27). We have demonstrated that SPARC is a newly identified factor secreted by the adipocyte and that its expression is strongly elevated in several models of experimental obesity. These observations suggest that elevated SPARC gene expression may be a general feature associated with these disorders. Further, we have shown that SPARC expression may be regulated in response to signals that modulate adipose tissue size such as insulin or high fat food. It would be now interesting to determine whether obesity in humans is also associated with altered expression of SPARC in the adipose tissue.

In an attempt to understand the physiological role of SPARC in the adipose tissue we studied the effect of SPARC on PAI-1 expression. Such a SPARC action has been previously described in bovine endothelial cells (35, 36). Similarly, we found that addition of SPARC to cultured whole adipose tissue induced an increase in PAI-1 mRNA levels. PAI-1 is the primary inhibitor of fibrinolysis, and its elevation has been associated with an increased risk for thrombotic disease (40). Up-regulation of PAI-1 expression by the adipose tissue is a major contributor to the elevation in plasma PAI-1 observed in obesity (11, 37, 38). The regulation of PAI-1 expression in the adipose tissue has been shown to be partly controlled by TNF-α and TGF-β1 (38, 39, 41). Here we show that SPARC could be a regulator of PAI-1 expression also in this tissue. More importantly, our findings suggest that overproduction of SPARC by the adipocyte could be an event that contributes to increased PAI-1 levels in conditions associated with obesity. Whereas the relationship between SPARC and PAI-1 production by adipose tissue has to be further analyzed, it is likely that aberrant expression of SPARC may contribute to the impairment of the fibrinolytic system observed in obesity. The mechanism(s) by which SPARC affects the expression of PAI-1 is at present unknown. One hypothesis is that SPARC could exert its effects through a TGFβ1-dependent pathway. Indeed, it was shown that SPARC regulates TGFβ1 expression in mesangial cells (24). However, we were unable to detect a change in TGFβ1 mRNA levels in our experimental system and under the conditions tested (data not shown).

Because of the multitude of functional properties attributed to SPARC, it is possible that SPARC impacts on several cellular processes in the adipose tissue. It has been shown that SPARC can influence the expression and/or activity of specific proteins that regulate cellular interactions with the extracellular matrix (26, 28). For example, SPARC has been reported to bind many components of the extracellular matrix such as thrombospondin and certain types of collagen (26), and also to stimulate the production of matrix metalloproteinases (42, 43). In addition, SPARC possesses a strong anti-adhesive activity. Indeed, SPARC affects cell shape by disrupting focal contacts and lowering adhesion to the matrix and to neighboring cells (26, 28). These observations suggest that SPARC could increase matrix plasticity and facilitate adipose tissue remodeling. Another characteristic of SPARC is the binding of cytokines and growth factors, and modulation of cellular responses induced by these molecules (26, 28). In this regard, SPARC could have a modulatory action on the growth factors that mediate adipocyte hyperplasia and adipose tissue growth. Finally, a role of SPARC in the neovascularization of the adipose tissue should be considered. Angiogenesis is prerequisite for fat mass development (44). Although factors with angiogenic activities such as vascular endothelial growth factor (VEGF) and leptin are known to be secreted by adipocytes (45–47), the mechanisms that promote angiogenesis in obesity are largely unknown. Interestingly, SPARC has been implicated in the regulation of angiogenesis during wound healing and tumor growth (29). These observations are in favor of a role of SPARC in angiogenesis in the adipose tissue of obese mice.

In conclusion, SPARC is a newly identified autocrine and/or paracrine factor of the adipose tissue that may affect key functions of this tissue. We are currently addressing the physiological role of SPARC in the adipose tissue and its implication in pathological conditions of the adipose tissue such as adipocyte hypertrophy or hyperplasia, vascular remodeling, and development of obesity-associated complications.

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