

Original Article

Increased SPARC expression in skeletal muscle and adipose tissue of db/db mice

Hai-Yan Song¹, Xiao-Yao Yang¹, Lei Ding², Lu-Jie Yang¹, Hua Xie¹, Yan-He Wang¹

¹Department of Endocrinology and Metabolism, The Second Affiliated Hospital of Harbin Medical University, Harbin, China; ²Department of Basic Medical Science, Wuxi Medical School, Jiangnan University, Jiangsu, China

Received May 8, 2016; Accepted June 22, 2016; Epub August 1, 2016; Published August 15, 2016

Abstract: Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or basement membrane-40, is involved in osteogenesis, angiogenesis, wound healing, and tumorigenesis. SPARC is predominantly secreted by adipose tissue, which has been recently linked with insulin resistance and glucose metabolism. Skeletal muscle is responsible for more than 75% of glucose disposal in response to insulin. In the current study, we examined SPARC expression in insulin-resistant skeletal muscle and adipose tissue. Specifically, SPARC mRNA and protein levels were measured in adipose tissue and skeletal muscle of db/db and control groups. Increased SPARC expression was observed in adipose tissue and skeletal muscle of db/db mice, suggesting that the protein participated in insulin resistance and glucose uptake. Our findings collectively indicated that SPARC contributed to insulin resistance and regulation of glucose metabolism, supporting its utility in the prevention and treatment of type 2 diabetes mellitus (T2DM) and metabolic syndrome.

Keywords: Secreted protein acidic and rich in cysteine, AMP-activated protein kinase, insulin resistance, adipose tissue, skeletal muscle, glucose uptake

Introduction

Secreted protein acidic and rich in cysteine (SPARC) is the protein product of a single gene localized at the chromosomal region 5q31-33 which is closely proximal to the genes encoding macrophage colony-stimulating factor 1, interleukin 3, platelet-derived growth factor and β 2-adrenergic receptor [1]. SPARC, also known as osteonectin or basement membrane-40, is a member of the matricellular protein family, which was initially detected in bone and endothelial cells [2, 3]. The protein was subsequently identified in a variety of tissues during development, tissue repair and remodeling [4], including notochord, somites, embryonic skeleton, differentiating chondrocytes, megakaryocytes, and macrophages, at the sites of tissue injury [5]. Interestingly, SPARC is a multifunctional protein involved in osteogenesis, angiogenesis, wound healing, tumorigenesis, and pathogenesis of fibrosis of the kidney [6, 7] and liver [8]. The role of SPARC in modifying the ECM, particularly its effect on collagen produc-

tion and assembly, is well established [9, 10]. SPARC additionally contributes to collagen fibril formation in the dermis, and consistently, SPARC knockout mice display reduced collagen content in the dermis [11]. Incidentally, there is increasing evidence to support the involvement of SPARC in regulating the tumor microenvironment, tumor development, invasion, metastasis, angiogenesis, and inflammation [12].

SPARC is secreted by adipose cells, and its circulatory level is positively correlated with BMI in humans [13, 14]. Moreover, the protein is proposed to contribute to the pathogenesis of obesity-associated disorders, including T2DM and its complications, mainly through promoting insulin resistance. Although several activities are attributed to SPARC, little is known about its specific role in glucose metabolism in skeletal muscle. Intriguingly, SPARC overexpression in skeletal muscle has been reported, leading to the hypothesis that the protein plays a key role in the glucose transport system. However, this theory is yet to be confirmed.

Diabetes is associated with dysfunction of glucose metabolism and insulin resistance. The objective of this study was to assess the consequences of SPARC expression in skeletal muscle and adipose tissue, and ascertain whether SPARC contributed to insulin resistance and glucose metabolism.

Materials and methods

Materials

Ten-week-old male db/db mice and their heterozygote littermates were donated by the Model Animal Research Center of Nanjing University. The mice were housed in a temperature-controlled (21°C) room under a dark-light cycle of 12 h each. Following sacrifice, rat tissues were removed and stored at -80°C until further processing. This study was approved by the Animal Care and Handling Committee of Harbin Medical University.

Double antibody sandwich enzyme linked immunosorbent assay (ELISA)

The prepared serum was taken from the -80°C refrigerator, which underwent natural coagulation under the room temperature for 30 min, followed by centrifugation at 1000×g for 15 min under the room temperature. The sample serum was diluted by 40 times, and double antibody sandwich enzyme linked immunosorbent assay was employed for the determination. The absorbance value (OD value) of the standard and sample serum was read by the multi-functional micro-plate reader. The standard curve was plotted by the concentration and OD value of the standard serum, and SPARC concentration in the sample serum was calculated, which was finally multiplied by the dilution times.

Semi-quantitative RT-PCR

Specific primers were synthesized by Invitrogen, including SPARC forward, 5'-GAA GGT ATG CAG CAA TGA CAA CAA-3', and SPARC reverse, 5'-TTC GGT CAG CTC GGA ATC CA-3' (leading to amplification of a 160 bp SPARC DNA fragment), and β -actin forward, 5'-CCC AGC ACA ATG AAG ATC AAG ATC AT-3', and β -actin reverse, 5'-ATC TGC TGG AAG GTG GAC AGC GA-3'. Total RNA was extracted from adipose and skeletal muscle of

each sample using TRIzol reagent (Invitrogen). cDNA was generated from total RNA (1 μ g) using the Reverse Transcription system (Promega), and further amplified via 35 cycles of PCR using Tag PCR Master Mix (Takara) and specific primers. The reverse transcription reaction involved incubation at 42°C for 15 min, at 99°C for 5 min, and at 5°C for 5 min. Subsequent PCR reactions were performed under the following conditions: initial incubation at 95°C for 2 min; denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 15 s, 32 cycles; final extension at 72°C for 2 min. PCR products were analyzed by electrophoresis on 2% agarose gels. Band intensities were quantified using the gel documentation system.

Western blot analysis

Protein extracts (30 μ g) were separated by electrophoresis on the 10% SDS gel run at 100 V for 80 min. Fractionated proteins from sample homogenates were transferred to nitrocellulose membrane, as described previously. Membranes were blocked for 70 min in TBS-T (Tris-buffered saline with 0.1% Tween-20) containing 5% non-fat dry milk at the room temperature, and incubated overnight at 4°C with anti-SPARC and anti- β -actin primary antibodies (1:1000 dilution). Subsequently, membranes were incubated with the appropriate anti-rabbit secondary antibody diluted in TBS-T (1:4000) for 1 h. Proteins were visualized on Kodak film using an enhanced chemiluminescence (ECL) Western Blot Detection Kit according to the manufacturer's instructions.

Immunofluorescence analysis

Skeletal muscle and adipose tissues were fixed with 3% paraformaldehyde in PBS for 2 h. Sections were cut using a cryostat, and washed with 0.1 mol/l glycine in PBS for 30 min, permeabilized with 0.1 mol/l Triton X-100 in PBS for 10 min, and blocked with 5% goat serum in PBS for 4 h. Following overnight incubation at 37°C with primary antibodies (1:400), sections were washed three times with PBS, and subsequently incubated with FITC-conjugated goat anti-mouse secondary antibodies (1:200) for 90 min at the room temperature. Sections were washed for three times with PBS again, mounted in 500 ml/l glycerin with 10 mmol/l PBS

SPARC and db/db mice

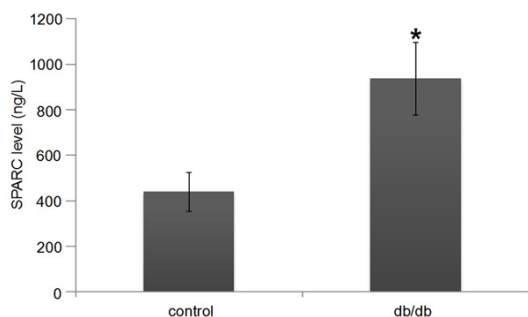


Figure 1. Plasma levels of SPARC. The graphs represent mean \pm SEM. * $P < 0.01$, compared to controls.

onto glass slides, and examined using an epifluorescence microscope with the appropriate filters and photographic attachment.

Results

Quantitative detection of serum SPARC concentration

The serum SPARC level in db/db mice was significantly higher than that in control mice ($P < 0.01$, **Figure 1**).

Expression levels of SPARC mRNA and protein

We analyzed both mRNA (**Figure 2A** and **2B**) and protein (**Figure 3A** and **3B**) expression levels of SPARC in skeletal muscle and adipose tissue in db/db mice, and compared them with the controls. Semi-quantitative PCR revealed elevation of SPARC mRNA in db/db mice compared to the controls. Western blot analysis was performed to ascertain whether there was increased expression of SPARC protein. SPARC protein expression data were consistent with those of semi-quantitative PCR

Immunofluorescence of SPARC

Immunofluorescence analysis for SPARC was performed in skeletal muscle (**Figure 4A**) and adipose tissue (**Figure 4B**). The distribution and intensity of fluorescence in these regions were increased in db/db mice, compared with the controls, indicating upregulation of SPARC expression.

Discussion

SPARC is closely linked with the elevated risk of metabolic disorders, especially T2DM and its

complications, which are characterized by insulin resistance. Previous studies have suggested possible involvement of SPARC in insulin resistance through regulating lipometabolism. Overexpression of SPARC has been reported in rodent models of diabetic nephropathy [15], and SPARC-null mice are protected against renal fibrosis [16]. Furthermore, SPARC expression is reduced in early diabetes-related kidney growth [17]. SPARC is expressed in retinal endothelial cells and associated with diabetic retinopathy [18]. In addition, diabetes-related mesenteric vascular hypertrophy is associated with increased SPARC expression in the vessel wall [19].

Insulin resistance in skeletal muscle and adipose tissue is linked to the development of metabolic diseases, such as obesity and hyperglycemia. Accordingly, recent research has focused on identifying natural bioactive compounds, with a view to improve insulin resistance associated with T2DM.

In the current study, we aimed to determine whether elevated SPARC expression was associated with insulin resistance and T2DM. Analysis of SPARC mRNA and protein expression levels clearly disclosed increased expression in adipose tissue and skeletal muscle of db/db mice compared with the controls.

Earlier investigations have shown that insulin resistance is derived from adipose tissue, leading to increased tissue decomposition and decreased synthesis. SPARC is an active component derived from adipocytes, which limits adipogenesis by inhibiting the differentiation of preadipocytes to mature adipocytes, suggesting its involvement in the regulation of adipocyte differentiation and insulin resistance. One possible mechanism is via stimulation of the wnt/ β -catenin signaling pathway, which triggers increased osteogenesis along with decreased adipogenesis. Adipogenic transcription factors, including CAAT/enhancer-binding protein α , CAAT/enhancer-binding protein β , and peroxisome proliferator-activated receptor γ , are significantly decreased by SPARC, along with adipocyte genes, such as leptin and lipoprotein lipase [20].

Skeletal muscle not only plays an important role in glucose homeostasis, providing an effective therapeutic target for T2DM, but also acts

SPARC and db/db mice

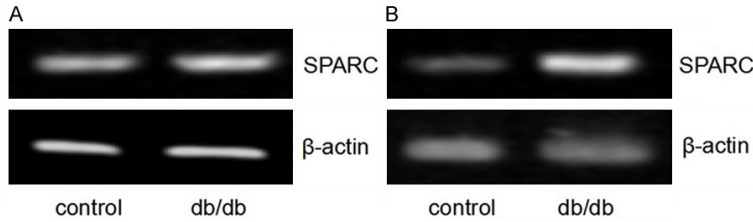


Figure 2. SPARC mRNA expression in skeletal muscle (A) and adipose tissue (B). Total RNA was extracted, and mRNA expression was evaluated using RT-PCR, with β -actin as the control. RT-PCR was conducted using specific primers. The amplified product was run on the 2% agarose gel and visualized with the gel documentation system.

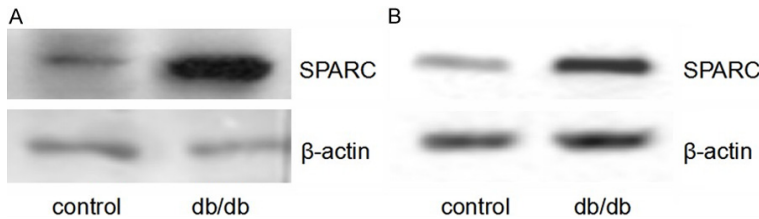


Figure 3. SPARC protein expression in skeletal muscle (A) and adipose tissue (B). Protein extracts were prepared for Western blot analysis, and SPARC expression was estimated by immunoblotting with the appropriate antibodies. The β -actin protein served as the control.

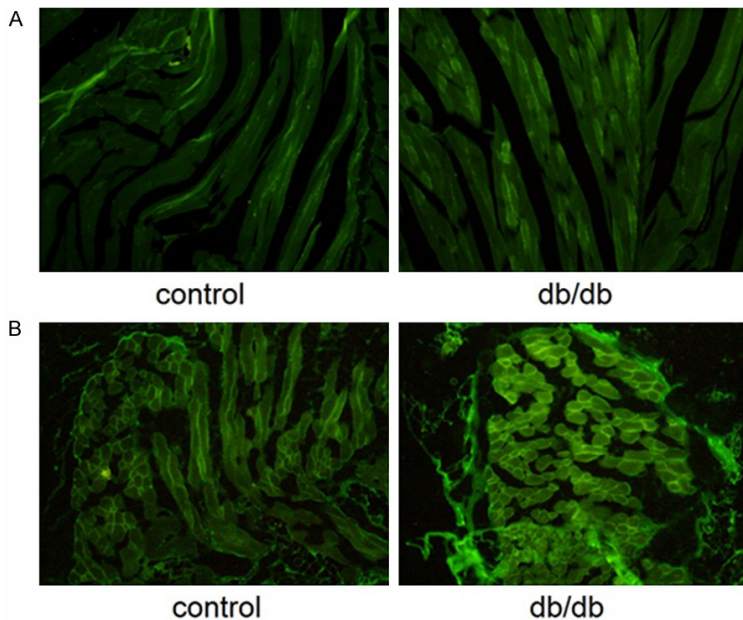


Figure 4. Immunofluorescence analysis of SPARC in skeletal muscle (A) and adipose tissue (B). Fixed tissues were permeabilized for 5 min in PBS containing 0.1% Triton X-100. Tissues were re-washed with PBS and incubated with mouse anti-human SPARC, followed by goat anti-mouse IgG-FITC. Distribution and intensity of fluorescence were increased in db/db mice compared with the controls.

as a major target of insulin. Thus it is commonly used for studying insulin resistance *in vitro*.

Notably, skeletal muscle is the major site of insulin-stimulated glucose utilization. The ability of SPARC inducing glucose uptake possibly depends on both the genetic makeup and metabolic properties of muscle. Glycogen phosphorylase activity exceeds glycogen synthesis activity in skeletal muscle, ensuring continued capacity for glycogenolysis. Data from the present study indicated that increased SPARC expression in skeletal muscles was associated with insulin resistance and T2DM.

Two possible mechanisms underlie SPARC involvement in insulin resistance and glucose metabolism, specifically, AMP-activated protein kinase (AMPK) and insulin signaling pathways. Previous studies have demonstrated that SPARC and AMPK co-interact with each other and enhance each other. SPARC may regulate glucose metabolism via AMPK activation [21]. AMPK plays a key role in sensing intracellular ATP levels, and acts as a crucial component in maintaining the energy balance within cells. Stimulation of AMPK triggers an increase in the intracellular AMP: ATP ratio [22]. Overexpression of SPARC may increase the intracellular AMP: ATP ratio, thus enhancing glucose uptake via AMPK activation. Recent findings have shown that expression and secretion of SPARC are influenced by glucose, insulin and leptin [13]. Skeletal muscle and adipose tissue represent the major sites of insulin-stimulated glucose utilization. Both PI3-kinase and AKT are linked to the insulin signaling pathway. Notably, SPARC promotes AKT and PI3-kinase phosphorylation in serum-free conditions, indicating stimulation of this pathway.

Since AMPK signaling is independent of insulin signaling [23], we hypothesize that cross-talk occurs between the two pathways.

In summary, our findings provide foundation for clarifying the relationship between SPARC and insulin resistance in T2DM, and extend our understanding about its roles in fat and glucose metabolism. We propose that SPARC presents an attractive agent for the treatment of metabolic disorders and prevention of insulin resistance in T2DM.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81370903 to Haiyan Song).

Disclosure of conflict of interest

None.

Address correspondence to: Hai-Yan Song, Department of Endocrinology and Metabolism, The Second Affiliated Hospital of Harbin Medical University, 246 Xuefu Road, Harbin 150080, China. Tel: +86-451-86297160; Fax: +86-451-86413857; E-mail: songhy@ems.hrbmu.edu.cn

References

- [1] Sage H, Johnson C and Bornstein P. Characterization of a novel serum albumin-binding glycoprotein secreted by endothelial cells in culture. *J Biol Chem* 1984; 259: 3993-4007.
- [2] Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML and Martin GR. Osteonectin, a bone-specific protein linking mineral to collagen. *Cell* 1981; 26: 99-105.
- [3] Saltman DL, Dolganov GM, Warrington JA, Wasmuth JJ and Lovett M. A physical map of 15 loci on human chromosome 5q23-q33 by two-color fluorescence in situ hybridization. *Genomics* 1993; 16: 726-732.
- [4] Lane TF and Sage EH. The biology of SPARC, a protein that modulates cell-matrix interactions. *FASEB J* 1994; 8: 163-173.
- [5] Tai IT and Tang MJ. SPARC in cancer biology: Its role in cancer progression and potential for therapy. *Drug Resist Update* 2008; 11: 231-246.
- [6] Reed MJ and Sage EH. SPARC and the extracellular matrix: implications for cancer and wound repair. *Curr Top Microbiol Immunol* 1996; 213: 81-94.
- [7] Bradshaw AD, Reed MJ and Sage EH. SPARC-null mice exhibit accelerated cutaneous wound closure. *J Histochem Cytochem* 2002; 50: 1-10.
- [8] Camino AM, Atorrasagasti C, Maccio D, Prada F, Salvatierra E, Rizzo M, Alaniz L, Aquino JB, Podhajcer OL, Silva M and Mazzolini G. Adenovirus-mediated inhibition of SPARC attenuates liver fibrosis in rats. *J Gene Med* 2008; 10: 993-1004.
- [9] Bradshaw AD. The role of SPARC in extracellular matrix assembly. *J Cell Commun Signal* 2009; 3: 239-246.
- [10] Seet LF, Su R, Barathi VA, Lee WS, Poh R, Heng YM, Manser E, Vithana EN, Aung T, Weaver M, Sage EH and Wong TT. SPARC deficiency results in improved surgical survival in a novel mouse model of glaucoma filtration surgery. *PLoS One* 2010; 5: e9415.
- [11] Bradshaw AD, Puolakkainen P, Dasgupta J, Davidson JM, Wight TN, Helene Sage E. SPARC-null mice display abnormalities in the dermis characterized by decreased collagen fibril diameter and reduced tensile strength. *J Invest Dermatol* 2003; 120: 949-955.
- [12] Chlenski A and Cohn SL. Modulation of matrix remodeling by SPARC in neoplastic progression. *Semin Cell Dev Biol* 2010; 21: 55-65.
- [13] Kos K, Wong S, Tan B, Gummesson A, Jernas M, Franck N, Kerrigan D, Nystrom FH, Carlsson LM, Randeve HS, Pinkney JH and Wilding JP. Regulation of the fibrosis and angiogenesis promoter SPARC in human adipose tissue by weight change, leptin, insulin and glucose. *Diabetes* 2009; 58: 1780-1788.
- [14] Takahashi M, Nagaretani H, Funahashi T, Nishizawa H, Maeda N, Kishida K, Kuriyama H, Shimomura I, Maeda K, Hotta K, Ouchi N, Kihara S, Nakamura T, Yamashita S and Matsuzawa Y. The expression of SPARC in adipose tissue and its increased plasma concentration in patients with coronary artery disease. *Obes Res* 2001; 9: 388-393.
- [15] Taneda S, Pippin JW, Sage EH, Hudkins KL, Takeuchi Y, Couser WG and Alpers CE. Amelioration of diabetic nephropathy in SPARC-null mice. *J Am Soc Nephrol* 2003; 14: 968-980.
- [16] Socha MJ, Manhiani M, Said N, Imig JD and Motamed K. Secreted protein acidic and rich in cysteine deficiency ameliorates renal inflammation and fibrosis in angiotensin hypertension. *Am J Pathol* 2007; 171: 1104-1112.
- [17] Gilbert RE, McNally PG, Cox A, Dziadek M, Rumble J, Cooper ME and Jerums G. SPARC gene expression is reduced in early diabetes-related kidney growth. *Kidney Int* 1995; 48: 1216-1225.
- [18] Munjal ID, McLean NV, Grant MB and Blake DA. Differences in the synthesis of secreted proteins in human retinal endothelial cells of diabetic and nondiabetic origin. *Curr Eye Res* 1994; 13: 303-310.
- [19] Jandeleit-Dahm K, Rumble J, Cox AJ, Kelly DJ, Dziadek M, Cooper ME and Gilbert RE. SPARC

SPARC and db/db mice

- gene expression is increased in diabetes-related mesenteric vascular hypertrophy. *Microvasc Res* 2000; 59: 61-71.
- [20] Nie J and Sage EH. SPARC inhibits adipogenesis by its enhancement of betacatenin signaling. *J Biol Chem* 2009; 284: 1279-1290.
- [21] Song H, Guan Y, Zhang L, Li K and Dong C. SPARC interacts with AMPK and regulates GLUT4 expression. *Biochem Biophys Res Commun* 2010; 396: 961-966.
- [22] Carling D. The AMP-activated protein kinase cascade-a unifying system for energy control. *Trends Biochem Sci* 2004; 29: 18-24.
- [23] Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ and Winder WW. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* 1999; 48: 1667-1671.