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Thrombospondin 1 Suppresses Insulin Signaling in C2C12 Myotubes

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Thrombospondin 1 (TSP-1) is abundantly expressed in visceral adipose tissue and this expression is up-regulated in obese humans and rodents. Recent studies showed that genetic deletion of TSP-1 protects mice from diet-induced insulin resistance. However, the molecular mechanism is largely unknown. In this study, we examined the effect of recombinant TSP-1 on insulin signaling in cultured cells from insulin sensitive tissues to investigate whether TSP-1 could act as an adipokine. Here we show that treatment with recombinant TSP-1 suppressed insulin signaling in cultured muscle cells, which was accompanied by the activation of stress signaling such as JNK, p38, and IKK. These results suggest that TSP-1 acts as an adipokine which is involved in the pathogenesis of obesity-induced insulin resistance. Thus, TSP-1 could be a potential target for the treatment of insulin resistance and metabolic disease related to insulin resistance.

INTRODUCTION

Obesity is a major risk factor for insulin resistance, which is a critical pathogenic factor in metabolic disease such as type 2 diabetes, dyslipidemia, hypertension, and coronary artery disease (21). White adipose tissue stores excess energy as triglyceride and provide free fatty acids as fuel to other tissues in response to energy status (24). In addition to the role in lipid handling, white adipose tissue plays an important role in the regulation of the production of a number of adipokines (24). Recent evidence revealed that dysregulated production of adipokines observed in obese adipose tissue contributes to the development of insulin resistance (18).

TSP-1 is a matricellular glycoprotein which was first characterized as a major component of platelet alpha granules (1,13). Since its initial description, TSP-1 has been found to be expressed in various cells (8,9,20). TSP-1 interacts with different ligands including extracellular matrixes, cell receptors, growth factors, cytokines, and proteases (2). Through these interactions, TSP-1 is thought to regulate a wide variety of physiological functions such as platelet aggregation, angiogenesis, cell adhesion, chemotaxis, and proliferation (2,22).

Recent studies revealed that genetic deletion of TSP-1 protects mice from diet-induced insulin resistance (14,26). Although these studies showed the involvement of TSP-1 in obesity-related adipose tissue inflammation and muscle fibrosis (14,26), the molecular mechanism has been largely unknown. In addition to existing as a component of extracellular matrix, TSP-1 is known to be secreted from many types of cells (2,9,19,23,25). Varma et al (23) showed that TSP-1 is secreted from adipocytes. Given that TSP-1 is expressed predominantly in visceral adipose tissue and the expression of this protein is up-regulated in obese humans and rodents (7,17), TSP-1 could be an adipokine associated with obesity and obesity related insulin resistance.

In this study, we examined the effect of recombinant TSP-1 on the activation of various pathways in cultured muscle cells and liver cells to investigate whether TSP-1 could act as an adipokine. Here we show that recombinant TSP-1 activates JNK, p38, and IKK pathway in these cells. Since the activation of these pathways is known to inhibit insulin signaling (21), we also investigated the inhibitory effect of recombinant TSP-1 on insulin signaling in cultured muscle cells or liver cells.

MATERIALS AND METHODS

Cell culture

C2C12 myoblasts and HepG2 cells were maintained in growth medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin) at 37°C with 95% air and 5% CO2. To induce differentiation of C2C12 myoblasts, growth medium was replaced with differentiating medium (DMEM containing 2% horse serum and 1% penicillin-streptomycin) when the cells reached confluence. All experiments were performed in fully differentiated C2C12 myotubes after 4 days in differentiating medium.

Recombinant Thrombospondin 1 treatment

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Recombinant human Thrombospondin 1 (TSP-1) was purchased from R&D systems (catalog no. TH-3074). The protein was reconstituted in sterile PBS and stored at -80°C and protected from exposure to light before using. C2C12 myotubes and HepG2 cells were serum starved for 16h and then treated with TSP-1 at the indicated concentrations.

Western blotting

The cells were lysed in 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1% TritonX, 2mM EDTA, 10% glycerol, and a protease inhibitor cocktail (Sigma, catalog no.P8340,1:100 dilution), a phosphatase inhibitor cocktail (Nacalai Tesque, catalog no.07575-51,1:100 dilution). Cell lysates were subjected to SDS-PAGE. Immunoblotting was performed using the following antibodies: Akt (Cell Signaling, catalog no. 9272), pAkt S473 (phospho-Akt Ser473) (Cell Signaling, catalog no.9271), pIKKα/β (phospho-IKKα/β) (Cell Signaling, catalog no.2078), IKKα/β (Santa Cruz Biotechnology, catalog no.7607), JNK (Cell Signaling, catalog no.9252), pJNK (phospsho-JNK) (Cell Signaling, catalog no.9251), pp38 (phospsho-p38) (Cell Signaling, catalog no. 9211), p38 (Cell Signaling, catalog no.9212), pERK (phopsho-ERK) (Cell Signaling, catalog no.9101), ERK (Cell Signaling, catalog no.9102), pIRS1 S636/639 (phospho-IRS1 Ser636/639) (Cell Signaling, catalog no.2388), and IRS1 (Millipore, catalog no.06-248).

Animal models

8-week-old male KKAy and C57BL/6J mice were purchased from CLEA Japan. Mice were maintained in a 12-h light/dark cycle and allowed free access to food (CE-2) and water. All experiments were performed according with the guidelines of the Animal Ethics Committee of Kobe University Graduate School of Medicine.

Tissue extraction and Quantitative real-time PCR analysis

KKAy and C57BL/6J mice were sacrificed by cervical dislocation. The following tissues were harvested from the mice and immediately frozen in liquid nitrogen: Brown adipose tissue (BAT), Epididymal white adipose tissue (epi WAT), Subcutaneous white adipose tissue (sub WAT), Liver, Gastrocnemius muscle (Muscle), Kidney, Pancreas, Lung, Heart, Brain. Total RNA was extracted from these tissues with RNeasy kit (Qiagen). The RNA samples were first converted into a complementary DNA (cDNA) using a reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) for real-time PCR analysis. Quantitative real-time PCR was performed with the StepOne Plus Real-Time PCR System (Applied Biosystems) using the Power SYBR Green PCR master mix (Applied Biosystems) and the specific primers. The expression of TSP-1 was normalized to that of 36B4. The primers used were as follows: TSP-1, 5′-GCAGCACACACAGAAGCATT-3′ (sense) and 5′-CAATCAGCTCTCACCAGCAG-3′ (antisense); and 36B4, 5′-GAGGAATCAGATGAGGATATGGGA-3′ (sense) and 5′-AAGCAGGCTGACTTGGTTGC-3′ (antisense).

Statistical analysis

All experiments were expressed as means ± SD. Statistical analysis was performed by 2-tailed Student's *t* test. Significance was accepted at $P<0.05$.

RESULTS

To study the mechanism by which TSP-1 contributes to the development of insulin resistance, we first examined mRNA expression of TSP-1 in various tissues of mice. TSP-1 was abundantly expressed in epididymal white adipose tissue while the expressions in other tissues were considerably lower than in white adipose tissue (Figure 1A). We next examined the alteration of TSP-1 expression in obese-diabetic KKAy mice. TSP-1 expression was significantly increased in epididymal white adipose tissue of KKAy mice compared to control mice (Figure 1B), which is consistent with the previous reports showing the increased expression of TSP-1 in white adipose tissue of obese humans and animals (7,17). These results indicate that the increase of TSP-1 expression in white adipose tissue might be involved in the development of insulin resistance.

TSP-1 has been shown to be a potential adipokine since this protein is secreted from cultured adipocytes into media (23). To study whether TSP-1 could act on skeletal muscle and liver, both of which are the major tissues in the regulation for glucose metabolism, we examined the effect of TSP-1 on the activation of various pathways in C2C12 myotubes or in HepG2 cells. C2C12 myotubes or HepG2 cells were treated with TSP-1 for 15 minutes and the activation of various pathways including JNK, p38, ERK, and IKK was examined by western blotting with phospho-specific antibodies. TSP-1 activated JNK, p38, and IKK in a dose-dependent manner in C2C12 myotubes (Figure 2A). ERK was not activated by TSP-1 treatment in C2C12 myotubes (Figure 2A). In HepG2 cells, TSP-1 activated JNK and p38 but not ERK and IKK (Figure 2B). These results suggest that TSP-1 activates stress signaling including JNK, p38, and IKK in cells from insulin sensitive tissues such as skeletal muscle and liver.

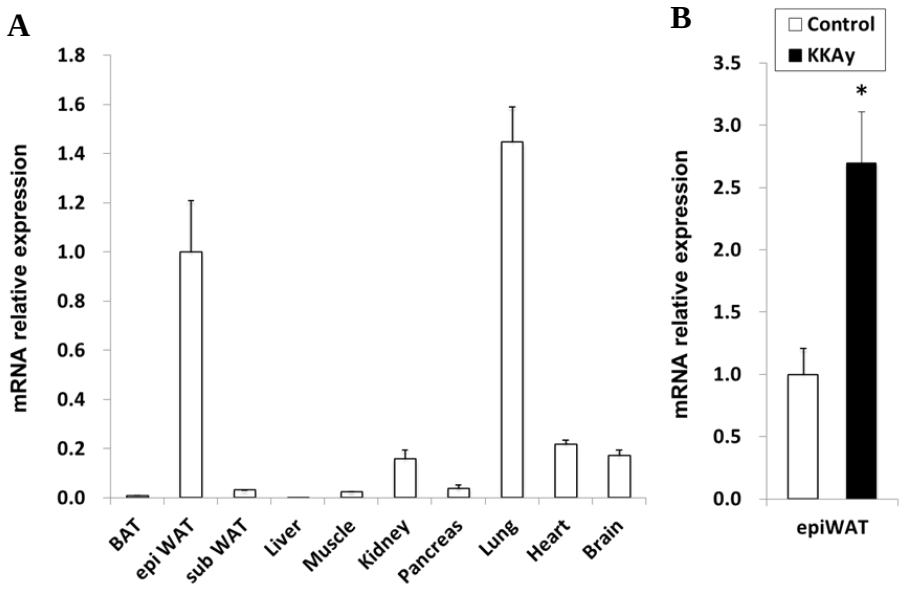

Figure 1. TSP-1 expression is up-regulated in epididymal white adipose tissue of KKAy mice.
(A) TSP-1 expression was examined in various tissues in C57BL/6J mice. n=5. (B) TSP-1 expression was examined in epididymal white adipose tissue (epi WAT) of C57BL/6J mice (Control) or KKAy mice (KKAy) at the age of 18 weeks. n=5 per group. * P<0.05

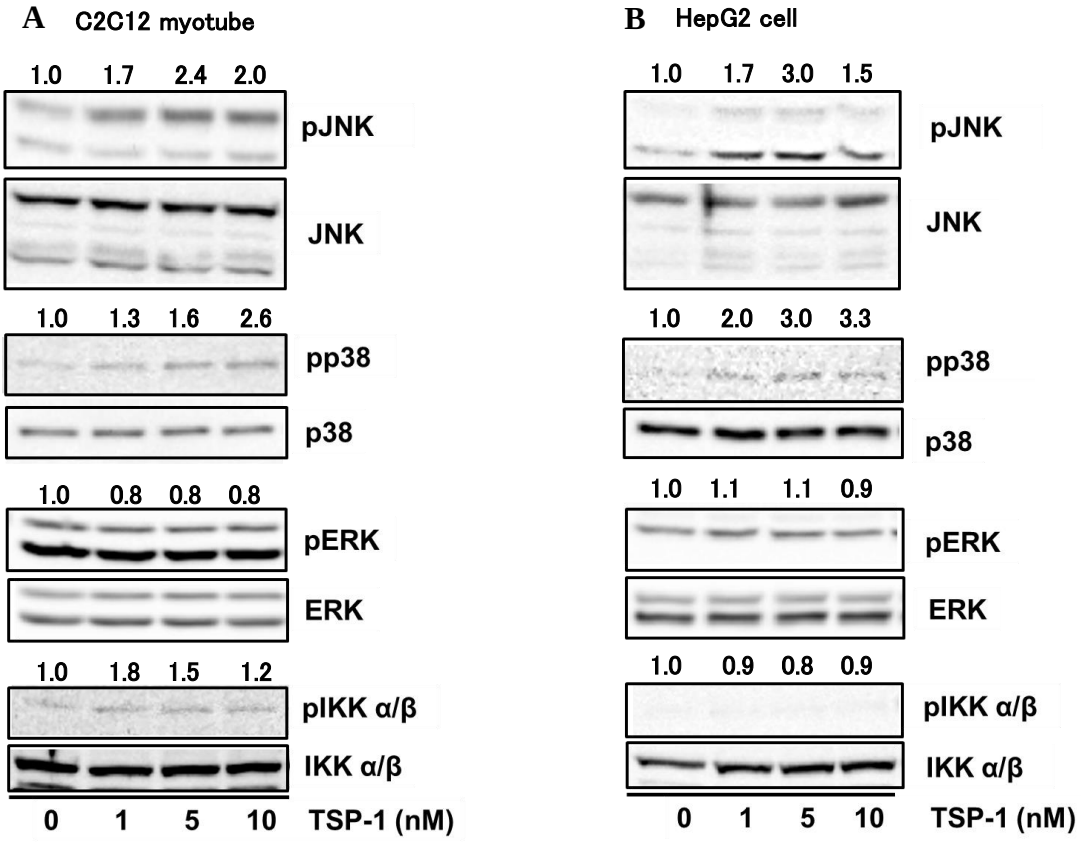

Figure 2. TSP-1 activates stress signaling in C2C12 myotubes and HepG2 cells.(A and B)
C2C12 myotubes (A) or HepG2 cells (B) were treated with TSP-1 at the concentration of 0, 1, 5, or 10nM for 15min, and western blotting was performed using the indicated antibodies. Representative immunoblots of JNK, pJNK, pp38, p38, pERK, ERK, pIKKα/β (pIKK), and IKKα/β (IKK) are shown. Fold increase to basal level is shown at the top of each panel.

Since the activation of stress signaling such as JNK, p38, and IKK induces serine phosphorylation of IRS1 to inhibit insulin signaling (21,27), we next examined whether TSP-1 affects the serine phosphorylation of IRS1 in

C2C12 myotubes and in HepG2 cells. TSP-1 increased the phosphorylation of IRS1 on Ser636/639 with a peak effect at 15 min in C2C12 myotubes (Figure 3A) while TSP-1 did not increase this phosphorylation of IRS1 in HepG2 cells (Figure 3B). Consistent with the results of serine phosphorylation in these cells, TSP-1 treatment significantly attenuated insulin-dependent Akt phosphorylation in C2C12 myotubes (Figure 4A) but not in HepG2 cells (Figure 4B). Suppressive effects of Akt phosphorylation by TSP-1 in C2C12 myotubes at insulin concentration of 10nM and 100nM were 16% and 26%, respectively (Figure 4A).

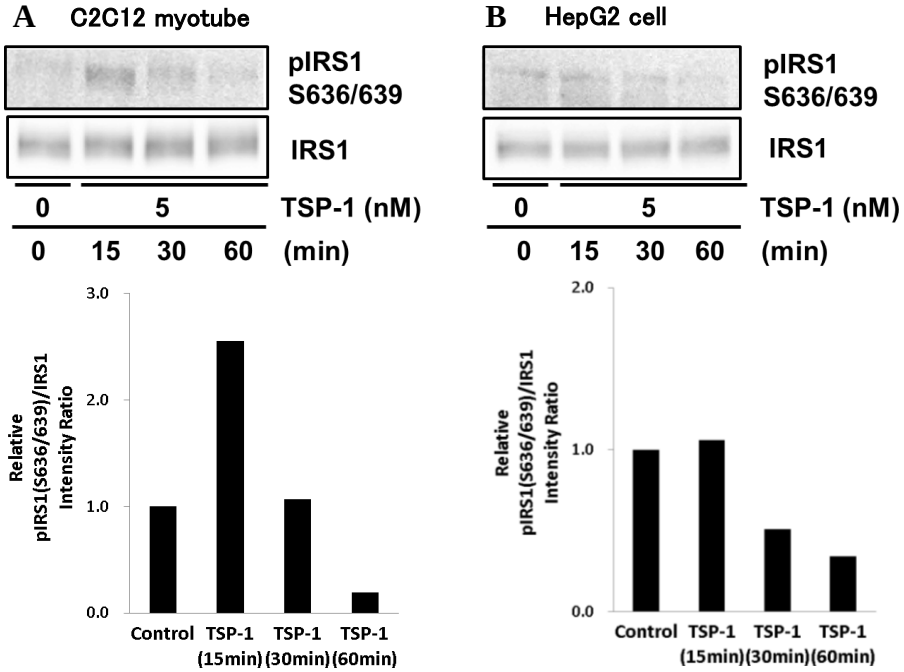

Figure 3. TSP-1 increases phosphorylation of IRS-1 on Ser636/639 in C2C12 myotubes.
(A) C2C12 myotubes were treated with or without TSP1 at a concentration of 5nM for 0, 15, 30, or 60min. Representative immunoblots of pIRS1 S636/639 and IRS1 are shown in upper panel. Quantification of immunoblots is shown in lower panel. (B) HepG2 cells were treated with or without TSP1 at a concentration of 5nM for 0, 15, 30, or 60min. Representative immunoblots of pIRS1 S636/639 and IRS1 are shown in upper panel. Quantification of the immunoblots is shown in lower panel.

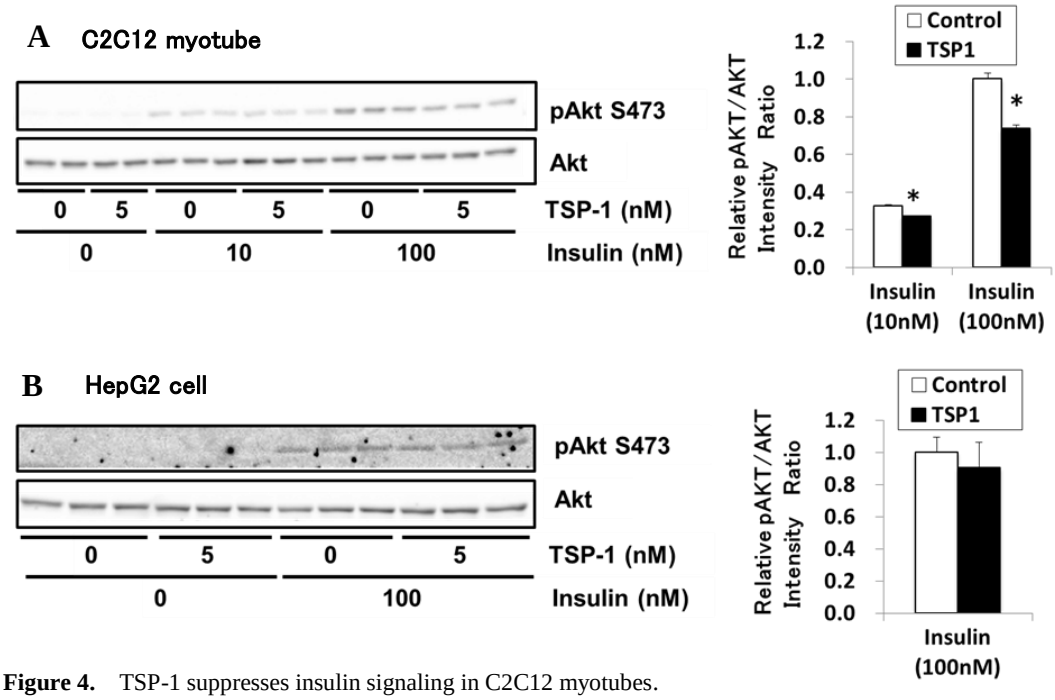

Figure 4. TSP-1 suppresses insulin signaling in C2C12 myotubes.
(A) C2C12 myotubes were treated with or without TSP1 at a concentration of 5nM for 60min and then stimulated by insulin (0, 10, or 100nM for 10min). Representative immunoblots of pAkt S473 and Akt are

shown in left panel. Quantification of the immunoblots is shown in right panel. n=3. (B) HepG2 cells were treated with or without TSP1 at the concentration of 5nM and then stimulated by insulin (0, 100nM for 10min). Representative immunoblots of pAkt S473 and Akt are shown in left panel. Quantification of the immunoblots is shown in right panel. n=3. * $P<0.05$

DISCUSSION

Recent studies showed that genetic deletion of TSP-1 protects mice from insulin resistance induced by high-fat diet (14) although the molecular mechanism has been largely unknown. TSP-1 exists both as a component of extracellular matrix and as a secreted form (22). It has been reported that TSP-1 is secreted from a wide variety of cells including adipocytes (2,9,19,23,25). Our study showed that TSP-1 is expressed predominantly in visceral adipose tissue and the expression of this protein is up-regulated in this tissue of obese-diabetic KKAy mice, which is consistent with the previous reports showing increased expression of this protein in obese humans and rodents (7,17). All these data indicate that TSP-1 might act as an adipokine associated with obesity and obesity-induced insulin resistance. We investigated this possibility using recombinant TSP-1 in vitro, and revealed that the secreted form of TSP-1 inhibits insulin signaling associated with the activation of stress signaling including JNK, p38, and IKK in muscle cells. Our results suggest that TSP-1 could be an adipokine involved in the pathogenesis of obesity-related insulin resistance.

We showed that TSP-1 has a potency to activate stress signaling such as JNK, p38, and IKK in cultured cells derived from insulin sensitive tissues although we didn't identify what receptor was responsible for this effect. TSP-1 interacts with a number of molecules including cell surface receptors (22). Among these molecules, TSP-1 is known to bind to CD36, a glycosylated protein member of the class B scavenger receptor family (12). CD36 plays an important role in the regulation of fatty acid and glucose metabolism (4). Binding with TSP-1, CD36 has been shown to activate p38, JNK, and IKK pathway in vascular endothelial cells and macrophages (10,11,15,16). CD36 is therefore one of possible candidates for the effect of TSP-1 in muscle and liver cells.

JNK, p38, and IKK are kinases which play important roles in relaying stress signaling (3). The activation of these kinases is known to inhibit insulin signaling through serine phosphorylation of IRS proteins (21,27). We observed that acute treatment of TSP-1 effectively attenuated insulin-dependent Akt phosphorylation associated with the increased IRS1 serine phosphorylation in cultured muscle cells. We showed that TSP-1 significantly attenuated insulin signaling in C2C12 myotubes whereas such attenuation by TSP-1 couldn't be observed in HepG2 cells. This contrast might be attributed to the difference in the sensitivity of IRS1 serine phosphorylation to stress signaling including JNK, p38, and IKK between cell types through unknown mechanism. Another possibility is the difference in the effect of TSP-1 on IKK activity. We observed that TSP-1 increased IKK phosphorylation in C2C12 myotubes but not in HepG2 cells. Since IKK plays a central role in modifying serine phosphorylation of IRS (5,6), the difference in IKK activity might contribute to the different effect of TSP-1 between these cell.

In conclusion, our study demonstrated that the secreted form of TSP-1 has a potency to suppress insulin signaling in cultured muscle cells associated with the activation of stress signaling such as JNK, p38, and IKK. TSP-1 was expressed predominantly in visceral adipose tissue and the expression was up-regulated in obese adipose tissue. All these results indicate that TSP-1 act as an adipokine which contributes to the development of obesity-induced insulin resistance. TSP-1 could be a therapeutic target for the treatment of insulin resistance and metabolic disease related to insulin resistance.

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