Peroxisome Proliferator-Activated Receptor γ Enhances Adiponectin Secretion via Up-Regulating DsbA-L Expression

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Running Title: PPAR γ promotes adiponectin secretion via DsbA-L

Keywords: Adiponectin, secretion, DsbA-L, PPAR γ

Document Figures & Tables Count: 5 Figures & 3 Tables

Contract grant sponsor (Contract grant number): National key Basic Research Program of China(2012CB124702); 948 Program (2013-S15), Specialized Research Fund for the Doctoral Program of Higher Education (20110146130002), Program of National Natural Science Foundation of China

21 (31172093, 30970356), and the National Science Foundation for Fostering Talents in Basic Research

22 (J1103510)

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Abstract

Disulfide-bond A oxidoreductase like-protein (DsbA-L) was identified as a molecular chaperone facilitating the assembly and secretion of adiponectin, an adipokine with multiple beneficial effects. In obesity the level of DsbA-L is reduced with a concomitant decrease of the circulating adiponectin level, especially of the high molecular weight form (HMW). Both rodent and human studies have shown that the nuclear receptor peroxisome proliferator-activated receptor (PPAR)- γ agonists increase adiponectin levels in serum by activating PPAR γ , which up-regulates critical endoplasmic reticulum (ER) chaperones thus facilitating protein folding. As shown in the present study, overexpression of PPAR γ in human embryonic kidney (HEK) 293 cells elicited the cellular release of HMW adiponectin. PPAR γ enhanced expression of DsbA-L by binding directly to peroxisome proliferator response element (PPRE) site within the DsbA-L promoter. Conversely, in differentiated 3T3-L1 cells, PPAR γ knockdown resulted in decreased expression of *Adiponectin*, *DsbA-L* and *ERp44*. *DsbA-L* expression increased after PPAR γ agonist treatment and decreased upon treatment with PPAR γ antagonist in 3T3-L1 adipocytes. DsbA-L deficiency in differentiated 3T3-L1 cells impaired the secretion of adiponectin. We therefore propose that DsbA-L plays an important role in facilitating HMW adiponectin formation and release from cells under the regulation of PPAR γ .

Introduction

Adiponectin is known for its roles in obesity-related disorders such as insulin resistance, type 2 diabetes mellitus, and atherosclerosis (Brochu-Gaudreau, Rehfeldt, Blouin et al., 2010). It is predominantly secreted by adipose tissue, and reaches a serum concentration of 2 to 10 $\mu\text{g/mL}$ (up to 30 $\mu\text{g/mL}$ in human) (Brochu-Gaudreau et al., 2010, Scherer, 2006). Adiponectin in circulation consists of at least three forms: trimeric (≈ 67 kDa), hexameric (≈ 140 kDa) and HMW (>300 kDa). Each multimeric form exhibits diverse biochemical properties and performs unique biological functions (Trujillo and Scherer, 2005, Kadowaki, Yamauchi, Kubota et al., 2006).

In the endoplasmic reticulum (ER), secretory and transmembrane proteins fold into their native conformation and undergo posttranslational modifications, which are important for their tertiary structures and functions (Schroder and Kaufman, 2005). Abundant protein chaperones residing in the ER lumen yield an oxidizing environment that is necessary for various secretory proteins such as adiponectin (Marciniak and Ron, 2006). Disulfide bond formation involving Cys39 in mouse adiponectin is vital for the stable production of hexamers and HMW multimers (Schraw, Wang, Halberg et al., 2008). The intratrimeric disulfide bond formation can be stimulated by the ER chaperones, such as ERp44, Ero1- α , and DsbA-L. Yet the formation of a disulfide bond in the inter-trimer or inter-hexamer mediated by the free Cys39 is facilitated by those chaperones (Wang and Scherer, 2008, Wang, Schraw, Kim et al., 2007).

The nuclear receptor PPAR γ can be perceived as a “master” regulator in fat cell differentiation and functioning (Kershaw and Flier, 2004). As ligands of PPAR γ , TZDs can improve both insulin sensitivity and circulating level of HMW adiponectin (Pajvani, Hawkins, Combs et al., 2004). However, the

mechanism of the formation and multimerization in chaperones mediating adiponectin disulfide bond remains unclear.

DsbA-L, which was identified as an adiponectin interacting protein, contains two thioredoxin-like domains and a conserved sequence (¹⁶Ser-Pro-Tyr-¹⁹Ser)(Liu, Zhou, Xu et al., 2008). It is a 25kDa protein sharing high sequence and secondary structure homology with *Escherichia coli* DsbA(Nebert and Vasiliou, 2004). Altering DsbA-L expression affects the multimerization and secretion of adiponectin in 3T3-L1 adipocytes. Treating 3T3-L1 adipocytes with Rosiglitazone increases the expression of both DsbA-L and adiponectin, resulting in the elevation of HMW adiponectin level(Liu et al., 2008).

In the present study, we examined whether PPAR γ mediated adiponectin secretion via up-regulating DsbA-L expression. We obtained the upstream sequence of the *DsbA-L* from *Mus musculus* from -1682 to +108, and identified a potentially functional peroxisome proliferator response element (PPRE) binding site in this region. We also found PPAR γ could upregulate DsbA-L by binding to its *cis*-acting element, thus enhancing the secretion of HMW adiponectin.

Materials and Methods

Isolating the 5'-flanking region of *Mus musculus* *DsbA-L*

Using the *Mus musculus* *DsbA-L* cDNA sequence as a query, a homologous genomic sequence was retrieved by Blast searches. Subsequently, primers were designed to specifically amplify the 5'-flanking

region of *Mus musculus DsbA-L*. The *DsbA-L* promoter was then cloned by PCR using mouse genomic DNA as the template. The PCR product was subject to sequencing, and the putative transcription initiation site and transcription factor binding sites were predicted using sequence alignment and MatInspector (<http://www.genomatix.de>).

Cell Culture and Differentiation

HEK293 cells were obtained from the Wuhan Cell Bank of the Wuhan University, and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. 3T3-L1 preadipocytes purchased from ATCC (The Global Bioresource Center in Hong Kong) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were grown to 90% confluence, before differentiation was started by the addition of 500 μ M isobutylmethylxanthine, 25 μ M dexamethasone, and 4 μ g/mL insulin for 2 days, followed by 2 days in supplemental media containing 4 μ g/mL insulin. Cells were cultured for an additional 2–6 days in supplemental media to complete differentiation. The PPAR γ agonist and antagonist used in the work are from Sigma(Shanghai, China).

Plasmid Construction and Silencer Vector Preparation

The coding sequences of mouse *DsbA-L* and the *DsbA-L* promoter were obtained according to the data from the NCBI gene database. The gene fragment generated by PCR was ligated in to pcDNA3.1 vector that had been digested by with *Bam*H I and *Hind*III, or the pGL3-Basic vector digested by *Kpn* I and *Xho* I. The target sequences for *DsbA-L* interference, obtained from Liu F's laboratory(Liu et al., 2008), and PPAR γ (GCCCTGGCAAAGCATTGTAT) were ligated in to the pSilencer4.1 CMV vector digested with *Bam*HI and *Hind*III. The sequences of all the primers used are shown in Table S1.

107

108 **Plasmid transfection**

109 In the overexpression experiment which compared adiponectin secretion in the HEK293 overexpressed
110 with PPAR γ /DsbA-L with that in the control cells, the transfection was carried in this way. First,
111 transfecting the 50% HEK293 with the same quantity of the pcDNA3.1-adiponectin, after 6h, the media
112 were replaced with DMEM with 10% fetal bovine serum. Then, after cultivation for 12 hours' time when
113 cells were grown to 80%, pcDNA3.1-PPAR γ /DsbA-L or pcDNA3.1 was transfected into different cells
114 groups separately. The transfection was done by Lipofectamine 2000 transfection reagent(Invitrogen). Six
115 hours later, the media were replaced with DMEM to collect the adiponectin secreted by HEK293 cells.

116

117 In the shRNA knockdown experiment in 3T3-L1 adipocytes, the pSilencer 4.1 CMV-DsbA-L/PPAR γ or
118 pSilencer 4.1 CMV-control was prepared to twice transfections. The transfection was done by
119 Lipofectamine 2000 transfection reagent (Invitrogen). After first transfection; opti-MEM was replaced
120 with DMEM with 10% fetal bovine serum for 12h cultivation. Then media were replaced with the
121 DMEM in 6h after the second transfection was conducted.

122

123 In the dual-luciferase reporter system experiment, the promoter plasmid, pGL3-Basic-DsbA-L, the
124 transcriptional plasmid, pcDNA3.1-PPAR γ and the pRL-TK were cotransfected into the HEK293 cells
125 due to the high transfection efficiency of HEK293. The transfection was done by Lipofectamine 2000
126 transfection reagent (Invitrogen). Six hours after cotransfection, the media was replaced with DMEM
127 with 10% fetal bovine serum for 24 h cultivation.

128

Quantitative RT-PCR Analysis

Extracted RNA was quantified to ensure a concentration of 3 µg/50 uL per cDNA reverse transcription reaction mixture including 4uL T18, 4uL dNTP, 10 uL reverse buffer and 1 uL of each M-MLV reverse transcriptase and RNasin Plus RNase inhibitor. Real time qPCR was performed using a CFX96 real time PCR system (Bio-Rad, Hercules, CA, USA) with SYBR Green PCR Mixture (Bio-Rad). The primers used for qRT-PCR are shown in Table S2. The PCR conditions were as follows: 95°C for 3 min, followed by 45 cycles of 95°C for 10 sec, and 60°C for 10 sec. The gene expression levels were normalized to *36B4*.

Western blotting analysis

The cells were lysed in a lysis buffer containing a proteinase inhibitors cocktail. The lysates were clarified by centrifugation at 12,000g for 5 minutes, before the supernatant was diluted with 5x native loading buffer. For denatured samples, mercaptoethanol was added to the samples, which were then boiled at 100 °C for 10 minutes, and subjected to SDS-PAGE. All proteins were transferred to a polyvinylidene fluoride membrane using transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, 0.01 % SDS, and 15% methanol using a LiuYi DY CZ-40D electrophoresis transfer cell to which 150 mA were applied for 180 minutes. Membranes were blocked using 5% non-fat dry milk, 20 mM Tris, pH 7.5, 500 mM sodium chloride, and 0.5% Tween-20 (TBS-T) for 1 hour. Individual proteins were incubated with primary antibodies at appropriate dilutions. Sources of antibodies (shown in Table S3) are as follows: PPARγ, Ero1-*La* and ERp44 monoclonal antibody from Cell Signaling Technology (Cell signal, MA), the adiponectin monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA) and DsbA-L polyclonal antibody from Proteintech (Proteintech, Wuhan). HRP-conjugated secondary antibodies (Santa Cruz) were incubated with the membrane at a dilution of 1:5000. Signals were detected using a

chemiluminescence detection system (MF-ChemBIS Bio-Imaging Systems; DNR Bio-Imaging Systems LTD). Equivalent protein loading between the samples was verified by blotting membranes for β -actin. The quantity analysis of the results from western blotting was made by Quantity One.

Luciferase reporter assay

Twenty-four hours after transfection, cells were lysed and assayed for promoter activity using the dual-luciferase reporter assay system. The enzymatic activity of luciferase was measured using an illuminometer (GloMaxTM 96 Microplate; Promega, WI).

Chromatin immunoprecipitation (ChIP) assay

3T3-L1 cells cultivated in a 100 mm culture dishes were differentiated for 8 days, and in cross linking using 1% formaldehyde for 10 min at room temperature. 12 weeks old C57BL/6 mice were from the Wuhan Center for Disease Control and Prevention, ChIP performed immediately after subcutaneous adipose tissue was obtained. Chromatin was isolated after cell and nuclear lysis, and then sonicated to generate 500-1000 bp DNA fragments. Anti-PPAR γ or rabbit IgG control antibodies were then used to immunoprecipitated the cross linked protein/DNA, before microtubes were mixed with suspended protein-G magnetic beads. Protein G bead-antibody/chromatin complexes were washed and eluted after incubating at 4°C overnight, and DNA was purified by spin columns. All reagents used in ChIP assays were from the ChIP assay kit (Millipore, Billerica, MA). Two pairs of primers (shown in Table S1) were prepared to amplify the region -480 bp to -179 bp and -1084 bp to -848 bp.

Statistical analysis

All experiments were repeated at least three times with the similar results. Data are given as the means $\pm$ sem. Student's *t* test was used for statistical comparison. $p < 0.05$ was considered as statistically significant.

Results

Overexpression of PPAR γ promotes adiponectin secretion

PPAR γ regulates many ER chaperones, and it is the dominant governor of adiponectin circulating levels *in vivo* (Xie, Tranchesi, Jia et al., 2008). However, how PPAR γ regulates adiponectin secretion remained unclear. HEK293 cells were co-transfected by pcDNA3.1-adiponectin and pcDNA3.1-PPAR γ or pcDNA3.1. Adiponectin protein levels in cell culture media were detected by SDS-PAGE western blotting. Results showed that the overexpression of PPAR γ in HEK 293 cells can increase adiponectin protein levels in the media, especially the HMW (high molecular weight) form (Fig. 1A, Fig. 1B). Concomitantly, the expression of DsbA-L, Ero1- α and ERp44 were increased in the cotransfected pcDNA3.1-adiponectin and pcDNA3.1-PPAR γ HEK293 cells, compared to the pcDNA3.1 control cells (Fig. 1C, Fig. 1D).

Pharmacological molecules targeting PPAR γ modulate adiponectin secretion

We differentiated 3T3-L1 adipocytes for 8 days, which were placed in exposure of a PPAR γ agonist (Rosiglitazone) or PPAR γ antagonist (GW9662) for 48h. As shown in Fig. 1E and Fig. 1F, western blotting for intact adiponectin revealed that Rosiglitazone increased the HMW in the media, while GW9662 had the tendency to reduce the HMW contents in the media.

PPAR γ increases *DsbA-L* expression

DsbA-L is a chaperone that interacts with adiponectin and facilitates the formation of the HMW form, yet the role of PPAR γ in *DsbA-L* transcription is unknown. HEK293 cells were nucleofected with pcDNA3.1-PPAR γ or pcDNA3.1, and the mRNA level of PPAR γ , *DsbA-L*, *Ero1-La* and *ERp44* were measured by qRT-PCR after 24h. We demonstrated that overexpression of PPAR γ led to increased PPAR γ , *DsbA-L*, *Ero1-La* and *ERp44* transcriptional levels in HEK293 cells (Fig. 2A). Differentiated 3T3-L1 cells were transfected with shRNA targeting PPAR γ or control nontargeting shRNA. PPAR γ knockdown reduced the expression of PPAR γ , adiponectin, *DsbA-L* and other chaperones. (Fig. 2B). Next, we treated 3T3-L1 preadipocytes with Rosiglitazone and/or GW9662. Rosiglitazone treatment increased *DsbA-L* mRNA level, while GW9662 inhibited *DsbA-L* expression (Fig. 2C). Same treatment in 3T3-L1 adipocytes with Rosiglitazone and GW9662 showed a consistent effect on *DsbA-L* mRNA level (Fig. 2D).

PPAR γ enhances *DsbA-L* promoter activity

In the mouse adipose cell line 3T3-L1, expressions of both PPAR γ and *DsbA-L* peak off after 8 days of differentiation (Fig. 3A, Fig. 3B). To confirm whether PPAR γ regulation of *DsbA-L* involved directly occupancy of PPAR γ on *DsbA-L* promoter, a 1790bp 5'-flanking region of *Mus musculus* *DsbA-L* was amplified by PCR, and HEK293 cells were then transfected with pGL3-Basic-*DsbA-L* to determine the effect of PPAR γ on *DsbA-L* promoter activity. Compared to pcDNA3.1 cotransfected HEK293, the luciferase activity of pGL3-Basic-*DsbA-L* increased after cotransfection with pcDNA3.1-PPAR γ (Fig.3C). Also, the data revealed that the luciferase activity of pGL3-Basic-*DsbA-L* increased 21 % after Rosiglitazone treatment in HEK293 cells (Fig. 3D). Several putative *cis*-acting elements were identified in the 5'-flanking region by MatInspector analysis on the 1790bp promoter sequence, including two PPAR sites, five RXR sites and two C/EBPs binding sites (Fig. 3E).

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220 **PPAR γ binds to the *DsbA-L* promoter**

221 According to the MatInspector analysis, truncated promoter pGL3-DsbA-L (-857/+108) was obtained
222 without the PPRE (-888/-866) within the promoter. To determine the binding sites of PPAR γ within
223 *DsbA-L* promoter, HEK293 cells were then co-transfected with pGL3-DsbA-L (-1247/+108)/pGL3-
224 DsbA-L (-857/+108) and pcDNA3.1-PPAR γ or pcDNA3.1. The data showed that pGL3-DsbA-L (-
225 857/+108) contained the predicted PPRE (-199/-177), but co-transfection with pcDNA3.1-PPAR γ
226 increased luciferase activity of pGL-DsbA-L (-1247/+108), not pGL3-DsbA-L (-857/+108) (Fig. 4A). It
227 revealed that PPAR γ may bind to the putative PPRE (-888/-866) to enhance the promoter activity.
228 Modifying the some bases of this PPRE eliminated the promotion of PPAR γ on DsbA-L promoter activity
229 (Fig. 4B).

230

231 Chromatin Immunoprecipitation (ChIP) was applied to identify the exact PPRE in the *DsbA-L* promoter.
232 Chromatin, harvested after nuclear lysis, was immunoprecipitated by a PPAR γ -specific antibody. Based
233 on bioinformatics predictions, two pairs of primers (-1084/-848 and -480/-179) were used to detect the
234 possible PPRE sites. PCR amplification then identified -1084/-848 as the PPRE site of *DsbA-L* promoter
235 (Fig. 4C). And the same ChIP assay was performed using the subcutaneous adipose tissue from 12 weeks
236 old male mice (Fig. 4D). Consistent with previous data, -888/-866 was identified as the certain PPRE site
237 in this region, which was highly conserved among humans, mice, and rats (Fig. 4E).

238

239 **DsbA-L plays a key role in PPAR γ mediating adiponectin secretion**

Previous studies showed that endogenous adiponectin interacted with endogenous DsbA-L in 3T3-L1 adipocytes(Liu et al., 2008). Each of shRNA vectors targeting *DsbA-L* and *PPAR γ* was used to transfect 3T3-L1 cells being differentiated for 8 days. Thirty-six hours after transfection, media was collected to determine adiponectin secretion, and cells were lysed to measure intracellular adiponectin concentrations by western blotting. The efficiencies of the shRNA vectors, shPPAR γ and shDsbA-L, were measure by western blotting respectively. As shown in Fig. 5A and Fig.5B, compared to the shControl adipocytes, PPAR γ level decreased almost 65% in shPPAR γ cells, and in shDsbA-L cells of DsbA-L level decreased nearly 50%.

In shPPAR γ cells, there was a reduction in adiponectin secretion, especially the HMW form. A similar decrease has been found in shDsbA-L cells (Fig. 5C). In consistent with this, DsbA-L or PPAR γ silencing resulted in the elevated intracellular HMW adiponectin levels (Fig. 5D). In addition, PPAR γ knockdown in differentiated 3T3-L1 adipocytesresulted in a decrease in DsbA-L and ERp44 expression (Fig. 5E).

To identify the role of DsbA-L in adiponectin assembly and secretion, pcDNA3.1 or pcDNA3.1-DsbA-L and pcDNA3.1-adiponectin were cotransfected into HEK293 cells to simulate the adiponectin assembly in 3T3-L1, in spite of other chaperones. DsbA-L overexpression was detected by RT-PCR and Western blotting (Fig. 5F). Cell media was collected to detect the adiponectin complexes levels by SDS-PAGE western blotting. The data revealed that the HMW form of adiponectin level increased when DsbA-L and adiponectin were overexpressed in HEK293 cells compared with those where only adiponectin was overexpressed (Fig. 5G).

Discussion

The current study reveals a novel mechanism of action of the role of PPAR γ in adiponectin secretion. Our results establish that an important function of PPAR γ is to regulate adiponectin secretion and its secretion-related proteins. Further, when 3T3-L1 cells were treated with a PPAR γ agonist, the mRNA level of *DsbA-L* increased concurrently with elevated PPAR γ activity and adiponectin secretion, while treating cells with a PPAR γ antagonist resulted the opposite effects. Our results suggest that PPAR γ enhances *DsbA-L* promoter activity by binding directly to its PPRE (-888/-866), which explains the effect of PPAR γ on adiponectin secretion. PPAR γ knockdown results in a decreased secretion of HMW adiponectin as well as a down regulation of certain chaperons, including DsbA-L, ERp44 and Ero1-L α . Our results reveal that DsbA-L plays a vital role in adiponectin secretion that *DsbA-L* deficiency in differentiated 3T3-L1 adipocytes blocks the release of HMW adiponectin, and that DsbA-L overexpression facilitates HMW adiponectin secretion in HEK293 cells.

Although our data showed the effect of PPAR γ on increasing DsbA-L transcriptional level to enhance the secretion of HMW adiponectin, a few questions remained unclear. First, the effect of the pharmacological molecules targeting PPAR γ , especially GW9662, on the cellular concentration of adiponectin forms was not identical to that of shPPAR γ . One possible explanation for seemingly contradictory results was that the impact of GW9662 made is to not only reducing the activity of PPAR γ , but also affect the cellular condition which is unknown. The second contradictory is whether PPAR γ increases or decreases the transcriptional level of ERp44. It is demonstrated in our study that PPAR γ silencing led to the reduction of ERp44 mRNA level in 3T3-L1 adipocytes whereas Long verified that PPAR γ downregulates ERp44 using porcine-derived materials. We performed the luciferase reporter assay on mouse-derived promoter; similar results that PPAR γ enhanced the ERp44 promoter activity were obtained. The potential explanation is that the mechanism of PPAR γ mediating ERp44 in porcine is different from that in mouse. In addition, it does not make sense that, after modifying the conserved PPRE site, luciferase activity of the

mutant promoter was much higher than that of the wild type. Through thorough analysis of the results from MatInspector prediction, we found that the bases (GTTG) were essential for the four transcriptional factors including a repressor, which explains the higher promoter activity.

Previous studies revealed an interaction between DsbA-L and adiponectin that may cause a conformational change in adiponectin, favoring a disulfide bond exchange reaction with PDI(Wang and Scherer, 2008). Liu et al. demonstrated that DsbA-L overexpression increases both the intracellular level and secreted level of adiponectin(Liu et al., 2008), in particular, the HMW form. These observations were consistent with our findings that co-transfection of pcDNA3.1-DsbA-L and the mouse adiponectin vector augment HMW adiponectin in the media.

It has been demonstrated that DsbA-L can interact with adiponectin in 3T3-L1 adipocytes(Liu et al., 2008). Unlike other PDI members, the conserved domain CXXC is replaced by SXXS in DsbA-L. In addition, Ser¹⁶ plays an essential role in the activity of DsbA-L. It is possible that the hydroxyl groups in DsbA-L may prevent thiol groups from adjacent adiponectin from forming a disulfide bond, thus enhancing inter-trimer disulfide bonds formation. As shown in a study by Briggs et al(Briggs et al., 2011), the formation of adiponectin complex needs a moderate redox environment. But surprisingly, excessively oxidizing conditions trigger inter-trimer disulfide bond formation or the assembly of fully oxidized hexamers, which are unable to assemble further into HMW adiponectin ultimately(Briggs, Jones, Mashalidis et al., 2009). Here these chaperones together may play an important role in maintaining an appropriate ER environment to prevent intramolecular disulfide bond formation, thus ensuring the normal assembly of hexameric or HMW adiponectin.

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311 Originally cataloged as a member of the GST kappa family, DsbA-L (GSTK1) was predicted to have an
312 important role in lipid metabolism(Raza, 2011,Morel, Rauch, Petit et al., 2004). After its renaming in
313 2008, DsbA-L was shed more light on for its implication with adiponectin assembly and the observation
314 that its expression is negatively correlated with metabolic syndrome in humans(Zhou and Liu, 2010,Zhou,
315 Liu, Zhang et al., 2010). However, it remains unclear how, located in peroxisome and mitochondria(Raza,
316 2011,Morel et al., 2004,Petit, Michelet, Rauch et al., 2009), DsbA-L exerts its facilitation of adiponectin
317 secretion(Liu et al., 2008,Liu, Xiang, Wilk et al., 2012,Wang, Liu, Liu et al., 2011), which takes place in
318 ER, Golgi and vesicles. Dual fluorescence studies in HepG2 cells ruled out the possibility that DsbA-L
319 was located in the cytoplasm(Morel et al., 2004). Further work therefore needs to be done to explain this
320 contradiction. Moreover, its correlation with insulin resistance, obesity and diabetes has been confirmed
321 in recent studies(Shield, Murray, Cappello et al., 2010,Gao, Fang, Zhang et al., 2009). Interestingly,
322 GSTK1 (-/-) mice showed no difference in adiponectin secretion in serum in a large number of
323 mice(Theodoratos, Blackburn, Coggan et al., 2012). This is in contrast with our current study, where
324 DsbA-L silencing did decrease the secretion of HMW secretion. And its deficiency resulted in a series of
325 cellular homeostasis changes. More work are therefore needed to be done to verify the role of DsbA-L as
326 a potential target for therapeutic intervention in insulin resistance and related disorders.

327

328

329 It was demonstrated that differentiated 3T3-L1 cells undergo great pressure due to progressive lipid
330 loading and triglyceride accumulation(Newton, Cologna, Moya et al., 2011). Enlarged adipocytes produce
331 the highest levels of adipocyte-derived hormones and reactive oxygen species (ROS). Several studies

have showed that the ER is one of the crucial targets of ROS(Lewen, Matz and Chan, 2000). Under such circumstance, the elaborated mechanism was implicated in to ensure the secretory proteins correct fold and assembly. Previous study revealed that overexpression of DsbA-L prevents ER stress-induced and autophagy-dependent downregulation of adiponectin(Zhou and Liu, 2010,Zhou et al., 2010). In consistent with previous studies, silencing *DsbA-L* in differentiated adipocytes resulted in a remarkable decrease in the amount of HMW released in media. It is therefore possible that DsbA-L plays an essential role in alleviating ER stress, and thus facilitating adiponectin assembly and secretion.

In summary, this study provides strong evidences for an important role of DsbA-L in PPAR γ -regulated adiponectin secretion in adipocytes. Our work also revealed that PPAR γ regulates DsbA-L expression level. Nevertheless, the underlying mechanism of how DsbA-L regulates the secretion of adiponectin by the cooperation with other chaperones remains unknown. More light should be shed on the intracellular location of DsbA-L, and its precise function in the ER, mitochondria, and peroxisome. We propose that the effects of DsbA-L on maintaining organism homeostasis may be related to obesity, type II diabetes and some metabolic syndrome diseases.

Acknowledgements.

This work was supported by the grants from National key Basic Research Program of China (2012CB124702), 948 Program (2012-S13), Specialized Research Fund for the Doctoral Program of Higher Education (20110146130002), Program of National Natural Science Foundation of China (31172093, 30970356), the National Science Foundation for Fostering Talents in Basic Research, J1103510)

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Figure Legends.

Fig. 1 PPAR γ enhances adiponectin secretion. Transfected pcDNA3.1-PPAR γ with pcDNA3.1-adiponectin, or pcDNA3.1 with pcDNA3.1-adiponectin in HEK293 cells, media were completely changed with DMEM without fetal bovine serum for 48 h, and then the media were collected for Western blot without denaturation for three forms of adiponectin (A,B) and with denaturation for adiponectin secretion related proteins as indicated (C,D). E,F, Treat 3T3-L1 differentiating for 8 days with 10 μ M rosiglitazone (PPAR γ agonist) or 5 μ M GW9662(PPAR γ antagonist) for 48 h, extracellular collection for native samples were detected by SDS-PAGE western blotting. The data are present as means $\pm$ SEM from 3 independent experiments. *(P<0.05) Indicates significant difference in expression from control.

Fig.2 PPAR γ increases transcriptional level of DsbA-L. A. pcDNA3.1-PPAR γ vector or pcDNA3.1 was transfected into HEK293 (adiponectin overexpression cells) cells for 24h, *PPAR γ* , *DsbA-L*, *Ero1-L α* and *ERp44* expression were detected by qRT-PCR. All values represent the mean $\pm$ SEM from 3 independent experiments. *(P<0.05) Indicates significant difference in expression from control. B. shPPAR γ or shcontrol vector was twice transfected into the differentiated 3T3-L1 at 8 days, after 72h, mRNA expression of PPAR γ , adiponectin and adiponectin secretion related molecular chaperones, like DsbA-L, Ero1-L α and ERp44 were determined using real-time PCR. Data are normalized to *36B4* and are expressed as mean $\pm$ SEM from 3 independent experiments. *(P<0.05) Indicates significant difference in expression from control shRNA. C, undifferentiated 3T3-L1 cells were exposed to 10 μ M rosiglitazone (PPAR γ agonist) or 5 μ M GW9662 (PPAR γ antagonist). DsbA-L expression was detected by qRT-PCR. All values are normalized to *36B4* and are expressed as mean $\pm$ SEM from 3 independent experiments. *(P<0.05) Indicates significant difference in expression from control (1 μ L DMSO). D, DsbA-L expression were determined in the differentiated 3T3-L1 (8 days) with treatment of 10 μ M rosiglitazone

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or 5 μ M GW9662. Data are normalized to *36B4* and are expressed as mean $\pm$ SEM from 3 independent experiments. * ($P<0.05$) Indicates significant difference in expression from control (1 μ L DMSO).

Fig.3 PPAR γ up-regulates *DsbA-L* promoter activity. A, B. PPAR γ and *DsbA-L* expression was determined by real-time PCR in 3T3-L1 cells grown to confluence then induced to undergo adipogenesis. Total RNA was collected at day -2, 0, 2, 4, 6, 8 and 10 of differentiation. Data are normalized to *36B4* and are expressed as the means. C. The pGL3-Basic-*DsbA-L* (-1682/+108) promoter were cotransfected with pcDNA3.1 or pcDNA3.1-PPAR γ . CMV-driven *Renilla* luciferase reporter plasmid was used as an internal control to correct the transfection efficiency. Data are expressed as mean $\pm$ SEM from 3 independent experiments. * ($P<0.05$) Indicates significant difference in expression from control. D. The pGL3-*DsbA-L* (-1682/+108) promoter were transfected into HEK293 cells with 10 μ M rosiglitazone or 1 μ L DMSO. The luciferase vector with *DsbA-L* promoter was constructed and transiently transfected into HEK293 cells as described. *Renilla* luciferase reporter plasmid was used as an internal control to correct the transfection efficiency. Data are expressed as mean $\pm$ SEM from 3 independent experiments. * ($P<0.05$) Indicates significant difference in expression from control. E. The consensus sequences for the putative transcription factor binding sites are marked as bars. The transcription initiation site is designed as +1.C/EBPs, ccaat-enhancer-binding protein; PPAR γ , peroxisome proliferator-activated receptor γ , RXR, retinoid X receptor.

Fig.4 PPAR γ binds to the *DsbA-L* promoter. A, Truncated promoter pGL3-*DsbA-L* (-1247/+108) and pGL3-*DsbA-L* (-857/+108) were cotransfected with pcDNA3.1-PPAR γ or pcDNA3.1 into HEK 293 cells. 24 h later, luciferase reporter activity was measured. B, Wild-type or a mutant of the ERp44 promoter was transfected into HEK 293 cells with pcDNA3.1-PPAR γ , and the activity of the promoter was measured.

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The conserved PPRE domain of DsbA-L promoter was mutated as indicated. pcDNA3.1 plasmid transfected was used as a vector control. *Renilla* luciferase reporter plasmid was used as an internal control to correct the transfection efficiency. Data are expressed as mean $\pm$ SEM from 3 independent experiments. *(P<0.05) Indicates significant difference in expression from control. C. ChIP assays were conducted in the 3T3-L1 differentiated at 8 days, the binding of PPAR γ to PPRE region of the DsbA-L promoter was amplified by PCR using pairs of aimed primers. D. ChIP assays were conducted in the subcutaneous adipose tissue from 12 weeks old male mice, the binding of PPAR γ to PPRE region of the DsbA-L promoter was amplified by PCR using pairs of aimed primers. E. Conserved sequences of the PPRE site of DsbA-L gene promoter. The aligned region is conserved in the human, rat and mouse, the human and rat DsbA-L promoter sequences are from NCBI database.

Fig.5 DsbA-L plays an important role in facilitating adiponectin secretion. Twice transfected with shPPAR γ or shDsbA-L, media were completely changed with DMEM without fetal bovine serum for 36 h. Proteins lysates were collected from control and DsbA-L or PPAR γ knockdown differentiated 3T3-L1 cells, shDsbA-L (A) and sh PPAR γ (B) knockdown efficiency were measured by western blotting. Extracellular protein(C) and proteins lysates (D) collected from control and knockdown differentiated 3T3-L1 cells were detected for natural adiponectin. E, the protein levels of DsbA-L and ERp44 were measure in the shPPAR γ and shcontrol differentiated adipocytes. F, The transcriptional level and protein level of DsbA-l were detected in the pcDNA3.1 and pcDNA3.1-adiponectin or pcDNA3.1-DsbA-L and pcDNA3.1-adiponectin. G. Extracellular collections for native samples for three forms adiponectin were detected by SDS-PAGE western blotting. Data are expressed as mean $\pm$ SEM from 3 independent experiments. *(P<0.05) Indicates significant difference in expression from control.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5