Endoplasmic Reticulum (ER) Localization Is Critical for DsbA-L Protein to Suppress ER Stress and Adiponectin Down-regulation in Adipocytes*

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Meilian Liu‡§1, Hongzhi Chen‡†1,2, Li Wei§**, Derong Hu, Kun Dong†††, Weiping Jia**, Lily Q. Dong‡‡, and Feng Liu‡††‡‡3

From the ‡ María Syndrome Research Center, the Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China, the † Departments of Biochemistry and Molecular Biology, University of New Mexico Health Science Center, Albuquerque, New Mexico 87131, the Departments of Pharmacology and Biochemistry, and ‡§ Cellular and Structural Biology, University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, Texas 78229, and the ‡¶ Department of Endocrinology and Metabolism, Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth People’s Hospital, Shanghai 200025, China

Adiponectin is an adipokine with insulin-sensitizing and anti-inflammatory functions. We previously reported that adiponectin multimerization and stability are promoted by the disulfide bond A oxidoreductase-like protein (DsbA-L) in cells and in vivo. However, the precise mechanism by which DsbA-L regulates adiponectin biosynthesis remains elusive. Here we show that DsbA-L is co-localized with the endoplasmic reticulum (ER) marker protein disulfide isomerase and the mitochondrial marker MitoTracker. In addition, DsbA-L interacts with the ER chaperone protein Ero1-Lα in 3T3-L1 adipocytes. In silico analysis and truncation mapping studies revealed that DsbA-L contains an ER targeting signal at its N terminus. Deletion of the first 6 residues at the N terminus greatly impaired DsbA-L localization in the ER. Overexpression of the wild-type but not the ER localization-defective mutant of DsbA-L protects against thapsigargin-induced ER stress and adiponectin down-regulation in 3T3-L1 adipocytes. In addition, overexpression of the wild type but not the ER localization-defective mutant of DsbA-L promotes adiponectin multimerization. Together, our results reveal that DsbA-L is localized in both the mitochondria and the ER in adipocytes and that its ER localization plays a critical role in suppressing ER stress and promoting adiponectin biosynthesis and secretion.

Adiponectin is 30-kDa adipokine with anti-inflammatory, anti-insulin resistance, and anti-atherosclerotic properties (1–3). Adiponectin circulating in plasma exists in three major forms: trimer, hexamer, and high molecular weight (HMW)4 multimer (4–7). It has been shown that the complex distribution, rather than the total levels of adiponectin, is associated with improved insulin sensitivity in response to thiazolidinedione stimulation in mice and humans (8). Consistent with this finding, the HMW form of adiponectin has been considered as having a major functional role in regulating glucose homeostasis (9–11). In support of this, impairment in adiponectin multimerization affects both secretion and function of this adipokine and is associated with diabetes and hypoadiponectinemia (4, 6). These findings suggest that increasing the ratio of the HMW form rather than the total levels of adiponectin might provide an effective alternative therapeutic strategy.

Several molecules, including the ER membrane-associated oxidoreductase-1 (Ero1)-Lα, ERp44, and the disulfide bond A oxidoreductase-like protein or DsbA-L, have been identified as key regulators of adiponectin multimerization and secretion (7, 12, 13). Our previous study showed that DsbA-L promotes adiponectin multimerization in 3T3-L1 adipocytes (13). Subsequently, we demonstrated that fat-specific overexpression of DsbA-L promoted adiponectin multimerization and protected mice against diet-induced obesity and insulin resistance (14). Overexpression of DsbA-L in 3T3-L1 adipocytes protected adiponectin from endoplasmic reticulum (ER) stress-induced down-regulation (15), suggesting a potential mechanism by

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† Both authors contributed equally to this work.

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§ To whom correspondence should be addressed. E-mail: LiuF@uthscsa.edu.

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which DsbA-L promotes adiponectin biosynthesis and function. However, how DsbA-L suppresses ER stress and how DsbA-L promotes adiponectin multimerization remain unknown.

In the current study, we demonstrate that DsbA-L is localized not only in mitochondria but also in the ER. In addition, we show that impairment in ER localization disrupts the ability of DsbA-L to suppress ER stress and to promote adiponectin assembly and secretion. Our study demonstrates for the first time an important role of DsbA-L in the ER and uncovers a mechanism by which obesity leads to ER stress and subsequent metabolic diseases.

**MATERIALS AND METHODS**

*Reagents*—The following chemicals were used: thapsigargin (350–004, Alexis Biochemicals, San Diego, CA), MitoTracker Red CMXRos (Invitrogen), and antibodies for cell staining against PDI (ABR Affinity BioReagents, Golden, CO), calnexin (Sigma), and complex IV (Abcam, Cambridge, MA). Polyclonal antibodies against adiponectin and DsbA-L were generated as described before (13). Antibodies against GADD 153/CHOP, Ero1-Lα, and ERP57 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-β-tubulin was from Sigma-Aldrich.

*Cell Culture, Transfection, in Vitro Binding, Immunoprecipitation, and Western Blot*—The maintenance and/or differentiation of HEK293 and 3T3-L1 cells were performed as described in our previous studies (13, 14). Cell transfection was performed by electroporation using the Neon transfection system (Invitrogen). Cells lysates and tissue homogenates were prepared in ice-cold lysis buffer, and the general procedures used for co-immunoprecipitation, in vitro binding, and Western blot have been described (16).

*RNA Interference and Generation of DsbA-L-suppressed Cells*—The sense and antisense sequences of siRNA were chemically synthesized and ligated into the pSIREN-RetroQ (BD Knockout RNAi system; BD Biosciences). The sequences for siRNA and scrambled control are GCATGGAAGCAACCGAGAT and GCCGTAGCACTGGAAGAAA, respectively. 3T3-L1 stable cell lines containing the DsbA-L siRNA construct or a scrambled control were generated as described in our previous study (13).

*Site-directed Mutagenesis*—Myc-tagged DsbA-L mutants were generated by single-stranded site-directed mutagenesis (17). Briefly, the primer lacking the coding sequence of 2GPAPR⁶ on DsbA-L was applied to synthesize the complementary strand and amplified in *Escherichia coli* (primer sequence: 5′-GTAGAAGAGTTCTAGAAATCTTCTACA-3′). To avoid potential degradation by the DsbA-L siRNA in the DsbA-L-suppressed cells, a silencing mutation was made in the siRNA recognizing the region of the cDNAs encoding the wild type and the mutants of DsbA-L. All site-directed mutagenesis products were confirmed by restriction mapping and DNA sequencing.

*Confocal Image Study*—For endogenous DsbA-L staining, 3T3-L1 fibroblasts were differentiated into adipocytes. ER was stained by the ER marker PDI. Purified DsbA-L antibody was used to detect subcellular localization of DsbA-L. For exogenous DsbA-L staining, plasmids encoding RNAi-resistant wild-type or mutant DsbA-L were introduced into DsbA-L-suppressed differentiated adipocytes by electroporation using the Neon transfection system. Cells were re-seeded in 24-well plates and cultured for 24 h. ER was indicated by calreticulin. Myc antibody was used to detect subcellular localization of the Myc-tagged DsbA-L mutants. Confocal images were taken by an Olympus FV1000 confocal microscope in the image core facility of UTHSCSA.

*Cell Fractionation*—Cell fractionation studies were performed according to the procedure as described with some modifications (18, 19). In brief, ~5 × 10⁷ 3T3-L1 adipocytes were collected by trypsinization and rinsed twice with PBS containing 250 mM sucrose, 0.5 mM EGTA, 5 mM HEPES (pH 7.4). The cells were then lysed by passing through a 27-gauge needle 10 times. Post-nucleus fraction was collected after centrifugation at 700 × g for 10 min, followed by 18,000 × g centrifugation for 25 min. For mitochondria and ER isolation, the pellets were suspended in 0.8 M sucrose and then overlaid on a continuous sucrose gradient (1–2M). The sucrose gradient was obtained by centrifugation at 140,000 × g for 2 h to let ER and mitochondria separate and migrate to the layer of their densities. Different layers were extracted using a 20-gauge needle and 1-ml syringe, diluted in isolation buffer, and centrifuged at 18,000 × g for 30 min. All the fractionation steps were performed at 4 °C. Pure mitochondria (bottom layers) and ER fraction (top layers) were verified by Western blot to double-check any potential contamination.

*Immunofluorescence and Western Blot Studies*—Western blot studies were performed as described previously (13, 14). Quantification of the relative change in protein levels detected by Western blot (expressed as a percentage of control protein levels, arbitrarily set as 1.0) was performed by analyzing Western blots using the Scion Image Alpha 4.0.3.2 program (Scion Corp.) and was normalized for the amount of protein loaded in each experiment.

*Statistical Analysis*—Statistical analysis of the data was performed using analysis of variance. Statistical significance was set at *p* values of *p* < 0.05 and **p** < 0.01.

**RESULTS**

*DsbA-L Is Localized in the ER and Interacts with ER Chaperone Ero1-Lα in 3T3-L1 Adipocytes*—Mammalian DsbA-L was previously found to be localized in mitochondria (20–22). The findings that DsbA-L interacts with adiponectin and promotes adiponectin multimerization suggest that the protein may also be localized in the ER. To test this possibility, we examined the localization of DsbA-L by confocal immunofluorescence experiments. In agreement with the findings of others (20–22), DsbA-L displays a punctate localization pattern in cells (Fig. 1A), indicating an organelle localization of the protein. Considerable amounts of DsbA-L proteins were co-localized with the mitochondrial marker MitoTracker in 3T3-L1 adipocytes, as demonstrated by the yellow color in the superimposed image (Fig. 1A). A fraction of DsbA-L was co-stained with the ER marker PDI (Fig. 1A), indicating that some of the DsbA-L proteins are localized in the ER.

To further confirm the dual localization of DsbA-L, we performed cell fractionation studies.
DsBA-L could be found in gradient layers containing either pure mitochondria or pure ER, which was determined by Western blot with the ER- and mitochondria-specific markers calnexin and complex IV, respectively (Fig. 1B). Recent studies showed that the ER proteins Er1-Lα and Erp57 play critical roles in regulating adiponectin multimerization in adipocytes (7, 12). To elucidate the potential mechanism by which DsBA-L regulates adiponectin multimerization, we asked whether DsBA-L interacts with these proteins in cells. Pulldown experiments revealed that DsBA-L interacted with Er1-Lα but not Erp57 (Fig. 1C). Immunoprecipitation experiments revealed that endogenous DsBA-L interacted with endogenous Er1-Lα in 3T3-L1 adipocytes (Fig. 1D). Taken together, these results suggest that localization in the ER and consequent interaction with the ER chaperone in this organelle may be required for DsBA-L to promote adiponectin multimerization and secretion.

Deletion of N-terminal Amino Acid Residues Impairs ER Targeting of DsBA-L—*In silico* analysis using the Protein Prowler and the Predotar programs both predicted a putative ER signal peptide at the N terminus of DsBA-L (residues 1–12), which is highly conserved in DsBA-L among different species (Fig. 2A). To determine the potential ER localization signal, we generated a DsBA-L mutant lacking the first 6 amino acid residues and named it as DsBA-L<sup>AN</sup>. Confocal immunofluorescence experiments revealed that deletion of the first 6 amino acids greatly blocked DsBA-L co-localization with the ER marker calreticulin (Fig. 2B, middle panel), but had little effect on (or even increased) its co-localization with MitoTracker (Fig. 2C, bottom panel). Consistent with these findings, the DsBA-L<sup>AN</sup> mutant no longer interacted with adiponectin, which is localized in the ER in 293 cells (Fig. 2E), in intact 3T3-L1 adipocytes (Fig. 2D). DsBA-L<sup>AN</sup> mutation also blocked the interaction of DsBA-L with ER protein Er1-Lα (Fig. 2D) (7). To exclude the possibility that the mutation affects DsBA-L physical binding to adiponectin, we overexpressed Myc-tagged DsBA-L<sup>AN</sup> in 293 cells and purified the protein with an antibody to the Myc tag. *In vitro* binding experiments showed that deleting the N-terminal residues did not affect the direct binding of DsBA-L to adiponectin (Fig. 2F), suggesting that this mutation impairs DsBA-L localization in the ER, rather than the physical interaction of DsBA-L with adiponectin.

**Localization in the ER Is Critical for DsBA-L to Protect DsBA-L Suppression and Thapsigargin-induced ER Stress**—We recently found that overexpression of DsBA-L prevented thapsigargin-induced ER stress in 3T3-L1 adipocytes (15). To determine whether localization in the ER is necessary for the protective effect of DsBA-L, we expressed RNAi-resistant wild-type DsBA-L or the DsBA-L<sup>AN</sup> mutant in the DsBA-L-suppressed 3T3-L1 adipocytes, which show high levels of ER stress due to the lack of DsBA-L (15). Overexpression of the wild type but not the DsBA-L<sup>AN</sup> mutant of DsBA-L greatly reduced ER stress and adiponectin down-regulation in the DsBA-L-suppressed 3T3-L1 adipocytes (Fig. 3A). This result demonstrates that ER localization is critical for DsBA-L to suppress ER stress and adiponectin down-regulation. To further test this, we treated 3T3-L1 adipocytes transiently expressing the wild type or the DsBA-L<sup>AN</sup> mutant of DsBA-L with or without thapsigargin for 24 h. Thapsigargin treatment greatly induced ER stress and adiponectin down-regulation, as demonstrated by increased CHOP expression (Fig. 3A). Overexpression of the wild type but not the ER localization-deficient mutant of DsBA-L markedly reduced thapsigargin-induced ER stress and adiponectin down-regulation (Fig. 3A), which confirmed that ER localization of DsBA-L is essential for its protective effect on ER stress and ER stress-induced adiponectin down-regulation. Consistently, high fat diet feeding significantly induced the expression of ER stress marker CHOP in white adipose tissue *in vivo* (Fig. 3, B and C). Fat-specific overexpression of DsBA-L protected against high fat diet-induced CHOP expression, but had little effect on the expression of ER protein calnexin *in vivo* (Fig. 3, B and C).

**ER Localization of DsBA-L Promotes Adiponectin Multimerization**—ER is the organelle responsible for the translation, folding, and transport of membrane or secreted proteins such as adiponectin. To determine whether ER localization is required for DsBA-L to promote adiponectin biosynthesis, we transiently expressed the wild type and the ER localization-
deficient mutant of DsbA-L in DsbA-L-suppressed 3T3-L1 adipocytes, which show a marked decrease in adiponectin total levels and multimerization (13). Overexpression of the wild type but not the ER localization mistargeting mutant of DsbA-L significantly restored the intracellular levels of adiponectin and its secretion (Fig. 4, A and B). In addition, non-reducing gel electrophoresis experiments revealed that overexpression of the wild type but not DsbA-LΔN mutant increased the ratio of the HMW form to total levels of the adiponectin in both adipocytes and the medium (Fig. 4, C–F). Together, these results demonstrate that ER targeting plays an important role for promoting the effects of DsbA-L on adiponectin multimerization.

DISCUSSION

We have recently demonstrated that DsbA-L promotes adiponectin multimerization, a cellular process that takes place in the ER of adipocytes. However, how DsbA-L regulates adiponectin multimerization in cells remains largely unknown. In the current study, we show that DsbA-L is co-localized with the ER markers PDI and calreticulin. In addition, DsbA-L interacts with ER proteins such as adiponectin and Ero1-Lα, demonstrating the ER localization of DsbA-L in adipocytes. We also found that deletion of the N-terminal sequence impairs DsbA-L ER localization (Fig. 2B) and its interaction with adiponectin and Ero1-Lα in adipocytes (Fig. 2D), but not its physical interaction with adiponectin (Fig. 2F), suggesting that this mutation affects only DsbA-L ER localization but not its direct binding to adiponectin. In addition, overexpression of the wild type but not the ER localization-deficient mutant of DsbA-L suppresses ER stress and improves adiponectin biosynthesis and multimerization in adipocytes, indicating that DsbA-L localization in the ER is critical for its promoting effect on adiponectin biosynthesis in 3T3-L1 adipocytes. These findings provide important information on the mechanism by which DsbA-L regulates adiponectin multimerization in adipocytes.

Although DsbA-L shares high sequence and secondary structure homology to bacterial disulfide bond-A oxidoreductase (DsbA) (23), it does not contain a CXXC redox active motif like other disulfide oxidoreductases such as DsbA and TcpG (24). Instead, it contains a SXXS (Ser16 and Ser19) motif involved in the regulation of adiponectin multimerization (13). Consistent with this, DsbA-L itself was not sufficient to promote adiponectin multimerization in the presence of oxidative glutathione and trimeric adiponectin in vitro assay (13). On the other hand, DsbA-L prevents ER stress and ER stress-induced
Ero1-L and in mice (7, 12). It is possible that DsbA-L cooperates with another ER chaperone Ero1-L motif-containing ER chaperone that has been shown to promote adiponectin assembly and secretion in mature adipocytes rather than directly catalyzing the formation of intermolecular disulfide bond necessary for adiponectin multimerization. In this study, we also found that DsbA-L is co-localized with the mitochondrial marker MitoTracker (Fig. 1A). In addition, cell fractionation studies revealed high levels of DsbA-L in mitochondria (Fig. 1B). These results are consistent with previous findings that DsbA-L is localized in the matrix of mitochondria in hepatocytes (20–22). There is some evidence suggesting that some proteins may be localized in both ER and mitochondria. For example, the anti-apoptotic Bcl-2 proteins, which act on the mitochondrial membrane to trigger the intrinsic pathway of apoptosis, are also found to reside and function in mitochondria (23–25). However, although the finding that DsbA-L is localized in mitochondria suggests that the protein may play a role in the maintenance of mitochondrial integrity and/or function, it remains to be determined whether mitochondrial dysfunction contributes to DsbA-L deficiency-induced ER stress and adiponectin down-regulation. Nevertheless, the dual localization of DsbA-L suggests that this protein may play important roles in both ER and mitochondrial function, and potentially, the

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**FIGURE 3.** ER localization plays a critical role for DsbA-L to protect ER stress in adipocytes. RNAi-resistant wild type and the ER localization-defective mutant of DsbA-L were transiently expressed in DsbA-L-suppressed 3T3-L1 adipocytes. A, the effect of DsbA-L overexpression on thapsigargin- or DsbA deficiency-induced ER stress in cells was determined by Western blot using an antibody to adiponectin (ADPN), CHOP, or Hsp60. Data are representative of three experiments with similar results. B, 5-week-old DsbA-L transgenic and wild-type control mice were fed with 45% high fat diet for 16 weeks as described in our previous study (14). The expression levels of CHOP, calnexin, and Myc-DsbA-L in epididymal fat were determined using Western blot. ND, normal diet; HFD, high fat diet; O/E, overexpression; Endo, endogenous. C, CHOP expression shown in panel B was quantified. *, p < 0.05 (analysis of variance).

**FIGURE 4.** ER localization is essential for DsbA-L promoting adiponectin secretion and multimerization. Differentiated DsbA-L-suppressed cells were transiently overexpressed. A and B, in the wild type and the ER localization-deficient mutant of DsbA-L, the intracellular (A) and secreted (B) adiponectin (ADPN) levels were determined by Western blot using specific antibodies as indicated. β-Tubulin and IgG heavy chain were used as loading controls (Ctr) for lysates and the medium, respectively. Data in A and B are representative of three independent experiments with similar results. C and D, in the meantime, the intracellular (C) and (D) secreted adiponectin were separated by non-reducing gradient gel followed by immunoblotting using an antibody to adiponectin. To reduce the impact of total adiponectin level on the detection of three forms of adiponectin in the non-reducing gel, the same volume of cell or medium samples in panels A and B for control and ΔN groups was loaded, but only one-third of the volume of cell or medium samples in panels A and B for WT groups was loaded (C and D). E and F, the percentages of intracellular (E) and secreted (F) HMW adiponectin in total adiponectin level were determined by quantification using Scion Image Alpha 4.0.3.2 program (Scion Corp.). *, p < 0.05 (analysis of variance).

adiponectin down-regulation (13, 15), suggesting that DsbA-L may function as an ER chaperone for normal ER function, rather than directly catalyzing the formation of intermolecular disulfide bond necessary for adiponectin multimerization. In agreement with this, our present study demonstrates that DsbA-L interacts with another ER chaperone Ero1-L, a CXXC motif-containing ER chaperone that has been shown to promote adiponectin assembly and secretion in mature adipocytes and in mice (7, 12). It is possible that DsbA-L cooperates with Ero1-L and selectively promotes adiponectin multimerization. Because overexpression of the wild type but not the ER localization-defective mutant of DsbA-L prevents thapsigargin-induced ER stress and adiponectin down-regulation (Fig. 3A), it is also possible that DsbA-L may function as a chaperone to improve protein folding/assembly efficiency in the ER and thus reduce protein overloading-induced ER stress. Further studies are warranted to distinguish these possibilities.
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functional link between these two important organelles in cells. Further studies will be needed to test this possibility.

In summary, we provide evidence for the first time that DsbA-L is localized in the ER. In addition, we show that ER localization is critical for DsbA-L to suppress ER stress and promote adiponectin assembly and secretion. Taken together with our previous finding that DsbA-L expression levels are significantly reduced in adipose tissues of obese mice and human subjects (13), it is conceivable that down-regulation of DsbA-L may have a contributing role in mediating obesity-induced ER stress, insulin resistance, and metabolic diseases. Thus, increasing DsbA-L expression could provide a protective effect against obesity-induced ER stress and thus improve metabolic homeostasis in vivo.

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