

# DsbA-L Alleviates Endoplasmic Reticulum Stress–Induced Adiponectin Downregulation

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**OBJECTIVE**—Obesity impairs adiponectin expression, assembly, and secretion, yet the underlying mechanisms remain elusive. The aims of this study were 1) to determine the molecular mechanisms by which obesity impairs adiponectin multimerization and stability, and 2) to determine the potential role of disulfide-bond-A oxidoreductase-like protein (DsbA-L), a recently identified adiponectin interactive protein that promotes adiponectin multimerization and stability in obesity-induced endoplasmic reticulum (ER) stress and adiponectin downregulation.

**RESEARCH DESIGN AND METHODS**—Tauroursodeoxycholic acid (TUDCA), a chemical chaperone that alleviates ER stress, was used to study the mechanism underlying obesity-induced adiponectin downregulation in *db/db* mice, high-fat diet-induced obese mice, and in ER-stressed 3T3-L1 adipocytes. The cellular levels of DsbA-L were altered by RNAi-mediated suppression or adenovirus-mediated overexpression. The protective role of DsbA-L in obesity- and ER stress-induced adiponectin downregulation was characterized.

**RESULTS**—Treating *db/db* mice and diet-induced obese mice with TUDCA increased the cellular and serum levels of adiponectin. In addition, inducing ER stress is sufficient to downregulate adiponectin levels in 3T3-L1 adipocytes, which could be protected by treating cells with the autophagy inhibitor 3-methyladenine or by overexpression of DsbA-L.

**CONCLUSIONS**—ER stress plays a key role in obesity-induced adiponectin downregulation. In addition, DsbA-L facilitates adiponectin folding and assembly and provides a protective effect against ER stress-mediated adiponectin downregulation in obesity. *Diabetes* 59:2809–2816, 2010

**A**diponectin is an insulin sensitizer that plays a versatile role in the regulation of energy homeostasis and insulin sensitivity. The serum adiponectin levels are significantly reduced under the conditions of obesity, insulin resistance, and type 2 diabetes (1), yet the precise underlying mechanisms remain largely unknown.

Adiponectin is synthesized as a single polypeptide of 30 kDa and is then assembled in the endoplasmic reticulum

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Received 25 March 2010 and accepted 30 July 2010. Published ahead of print at <http://diabetes.diabetesjournals.org> on 10 August 2010. DOI: 10.2337/db10-0412.

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(ER), primarily into three species: trimer, hexamer, and high molecular weight (HMW) multimer (2–5). The different forms of adiponectin have been found to play distinct roles in the regulation of energy homeostasis (2–4,6). Several ER-associated proteins, including Ero1- $\alpha$ , ERp44, and GPR94, have recently been found to be involved in the assembly and secretion of higher-order adiponectin complexes (5,7,8). Impairment in adiponectin multimerization affects both secretion and function of this adipokine, and is associated with diabetes and hypoadiponectinemia (2,4).

ER is a eukaryotic organelle responsible for several specialized and important functions such as protein translation, folding, and transport of membrane or secreted proteins. Numerous protein chaperones are present in the ER lumen that yield an oxidizing environment necessary for correct folding and assembly of various membrane and secretive proteins such as adiponectin. In obesity, increased demand on ER function leads to ER stress and the unfolded protein response (UPR) to ensure that normal cell function and viability are maintained (9). However, whether and how ER stress plays a role in obesity-induced adiponectin downregulation remain to be established.

We recently identified an adiponectin-interacting protein named disulfide-bond A oxidoreductase-like protein (DsbA-L) (10). DsbA-L is expressed in various mouse tissues such as liver, kidney, pancreas, and heart, but the highest expression of this protein is detected in adipose tissue where adiponectin is synthesized and secreted (10). The cellular levels of DsbA-L are significantly reduced in adipose tissues of obese mice and human subjects. Overexpression of DsbA-L promotes adiponectin multimerization while reducing DsbA-L expression by RNAi markedly, and selectively reduces adiponectin levels and secretion in 3T3-L1 adipocytes (10). However, how DsbA-L regulates adiponectin multimerization and stability remains unknown.

In the present study, we show that alleviating ER stress enhances the cellular and plasma levels of adiponectin in both *db/db* mice and diet-induced obese mice. In addition, we demonstrate that inducing ER stress is sufficient to downregulate the cellular levels and secretion of adiponectin in 3T3-L1 adipocytes. Furthermore, overexpression of DsbA-L suppresses the inhibitory effect of ER stress on adiponectin multimerization and stability. Our studies demonstrate that ER stress is a key factor in obesity-induced downregulation of adiponectin and that increasing the cellular levels of DsbA-L improves adiponectin assembly and stability by suppressing the negative effect of ER stress.

## RESEARCH DESIGN AND METHODS

**Chemicals and antibodies.** The following chemicals were used: thapsigargin (TG) (350–004, Alexis Biochemicals, San Diego, CA), 3-methyladenine (3-MA)

(M9281, Sigma-Aldrich), tauroursodeoxycholic acid ([TUDCA] 580549, Calbiochem, Gibbstown, NJ), and G418 sulfate (30–234-CR, Cellgro, Manassas, VA). Polyclonal antiadiponectin and DsbA-L antibodies were generated as described before (10). Antibodies against  $\beta$ -actin, AMP-activated protein kinase (AMPK), phosphor-Thr<sup>172</sup>-AMPK, Acetyl-CoA carboxylase, phosphor-Ser<sup>79</sup>-Acetyl-CoA carboxylase, and anti-LC3 were from Cell Signaling Technology (Danvers, MA). Other antibodies used were: monoclonal antiadiponectin (MAB3608, Chemicon International), anti- $\beta$ -tubulin 2.1 (T4026, Sigma-Aldrich), and anti-GADD 153/CHOP (sc-7351, Santa Cruz Biotechnology, Santa Cruz, CA).

**Cell culture, differentiation and adenovirus infection.** The culture of C2C12 cells and the murine hepatocytes was described in our previous studies (11,12). The 3T3-L1 cells stably expressing enhanced green fluorescent protein (EGFP)-LC3 were generated by transfection of cells with the pEGFP-LC3 plasmid, a gift from Dr. Jean X. Jiang (University of Texas Health Science Center at San Antonio), and selection with 400  $\mu$ g/ml of G418. Independent clones were selected by limited dilution. Next, C2C12 and 3T3-L1 cell differentiation were performed as described in our previous studies (10,11). Adenoviruses encoding GFP and GFP plus wild type or S16A mutant of DsbA-L were described in our previous studies (10).

**Animal studies.** Male *db/db* mice and their lean controls (stock# 000642, 3–5 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were group-housed in a specific-pathogen-free facility at 22 to 24°C on a 12-h light/12-h dark cycle with the lights on at 8:00 A.M. The mice were fed with standard rodent chow, and all animals had access to water ad libitum. At 8 weeks of age, the mice were intraperitoneally injected with TUDCA (250 mg/kg) or equal volumes of vehicle (saline) twice daily (at 8:00 A.M. and 8:00 P.M.) for 26 days. Mice were then killed and mouse tissues were isolated according to the procedure described in our recent studies (13). For high-fat diet (HFD) experiments, 6- to 8-week-old C57BL/6 mice (10 mice per group) were fed with regular chow diet (Research Diets #D12328: 10.5 kcal% fat, 73.1 kcal% carbohydrate, and 16.4 kcal% protein) or HFD (Research Diets #D12330: 58.0 kcal% fat, 16.0 kcal% protein, and 26 kcal% carbohydrate) for 5 months. During the last 3 weeks of the HFD feeding period, the mice were intraperitoneally injected with TUDCA (250 mg/kg) or equal volumes of vehicle (saline) twice daily (8:00 A.M. and 8:00 P.M.). Mice were killed, and mouse tissues were isolated according to the procedure described in our recent studies (13). All animal procedures were approved by the University of Texas Health Science Center Animal Care and Use Committee.

**Insulin tolerance test.** The saline- and TUDCA-treated male *db/db* mice and lean control mice (8–10 mice per group) were fasted for 6 h followed by insulin injection (2 IU/kg animal body weight i.p.). Blood was obtained from the tail vein before (0 min) and after insulin injection (15, 30, 60, and 120 min). Glucose levels were measured using an automatic glucometer (Rightest GM300; Bionime Corp, San Diego, CA).

**Determination of adiponectin multimers.** Adiponectin multimers were determined by gel filtration fast-protein liquid chromatography with a Superdex 200 10/30 column (GE Healthcare Bio-Sciences, Piscataway, NJ), as described in our previous study (10).

**Immunofluorescence study and Western blot studies.** The immunofluorescence and Western blot studies were performed as previously described (10,14). The localization and expression of adiponectin were visualized with an Olympus IX 81 confocal microscope with a 40X, 1.35 NA oil immersion objective. 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 Corporation) and was normalized for the amount of protein loaded in each experiment.

**Quantitative real-time PCR.** Adiponectin mRNA was purified mRNA from cells using TRIZOL reagent (Invitrogen). Total RNA was reverse transcribed using SuperScript III First-Strand Synthesis Supermix (Invitrogen). The cDNA samples were amplified in duplicate in 96-microtiter plates (Applied Biosystems). Each PCR reaction (20  $\mu$ l total volume) contained: 10  $\mu$ l of SYBR Green PCR Master Mix (Applied Biosystems), 5 pmols of each primer, and 1  $\mu$ g of cDNA. The PCR primers for adiponectin were: 5'-CCC AAG GGA ACT TGT GCA GGT TGG ATG-3' and 5'-GTT GGT ATC ATG GTA GAG AAG AAA GCC-3'. The mRNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was quantified as an endogenous control, using the following primers: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. Real-time PCR reactions were carried out in an ABI PRISM 7,700 real-time PCR apparatus. Reactions without a template or without the reverse transcriptase were used as negative controls.

**Statistical analysis.** Statistical analysis of the data were performed using ANOVA. Statistical significance was set at *P* values of \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

## RESULTS

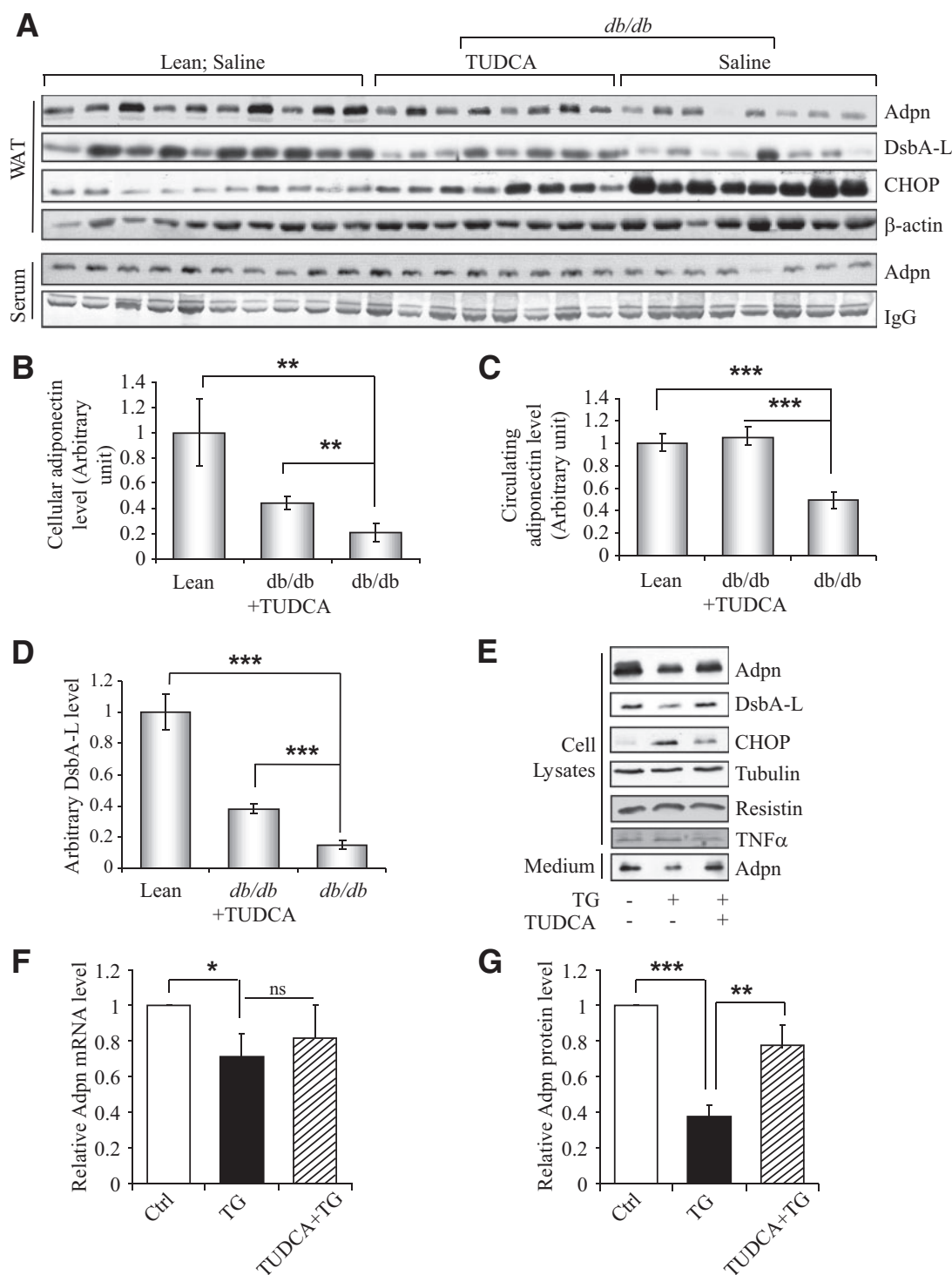
### Reduction of ER stress increases adiponectin levels in obese mice and in cells.

Obesity induces ER stress, and ER stress is correlated with reduced adiponectin levels (15). To determine whether ER stress is involved in downregulating adiponectin expression in obesity, we examined adiponectin levels in *db/db* mice treated with vehicle or the ER stress-reducing chemical chaperone, TUDCA. Obesity led to a great increase in ER stress in fat tissue of the *db/db* mice, as demonstrated by increased C/EBP-homologous protein (CHOP) expression (Fig. 1A). Concurrent with increased ER stress, the cellular (Fig. 1B) and circulating (Fig. 1C) levels of adiponectin were significantly reduced in the *db/db* mice compared with that of the lean mice. Treating the *db/db* mice with TUDCA greatly reduced obesity-induced CHOP expression, concurrent with an increase in circulating and cellular levels of adiponectin and its interacting protein DsbA-L (Fig. 1A–D). TUDCA treatment had no significant effect on the food intake (data not shown) (16) and body weight of the *db/db* mice (supplementary Fig. S1A in the appendix available online at <http://diabetes.diabetesjournals.org/cgi/content/full/db10-0412/DC1>, indicating that the TUDCA-mediated rescuing effect on DsbA-L and adiponectin levels was not a consequence of altered food intake/body weight. The expression levels of DsbA-L and adiponectin were also greatly reduced in WAT of HFD-induced obese mice (10) and this reduction was also rescued by TUDCA treatment (supplemental online Fig. S2). Taken together, these results suggest that ER stress may play a key role in obesity-induced adiponectin downregulation.

### DsbA-L prevents ER stress-induced adiponectin downregulation in 3T3-L1 adipocytes.

To determine whether ER stress is sufficient to reduce adiponectin levels, we examined adiponectin expression in 3T3-L1 adipocytes treated with and without thapsigargin (TG), a chemical widely used to stimulate ER stress by inhibiting ER calcium-ATPase (17). Treating 3T3-L1 adipocytes with TG led to a marked increase in ER stress, as demonstrated by enhanced CHOP expression (Fig. 1E). TG treatment also greatly reduced the cellular levels of adiponectin and DsbA-L as well as adiponectin secretion into the medium, which could be partially prevented by pretreating the cells with TUDCA (Fig. 1E). Although inducing ER stress by TG led to an ~25% reduction in the mRNA levels of adiponectin, treating 3T3-L1 adipocytes with TUDCA had no significant rescuing effect on the TG-induced downregulation of the mRNA levels of adiponectin (Fig. 1F). On the other hand, TG treatment led to a 60% reduction in the protein levels of adiponectin and the TG-induced downregulation of adiponectin was significantly protected by treating the cells with TUDCA (Fig. 1G), suggesting that the protective effect of DsbA-L on cellular levels of adiponectin is mainly at the protein level rather than mRNA level. TG treatment had no significant effect on the expression levels of two other adipokines, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and resistin (Fig. 1E), suggesting a selective role of this ER stress inducer in adipokine stability.

Since DsbA-L levels are negatively associated with obesity (10) and ER stress (Fig. 1), we were interested in determining whether reducing the expression levels of DsbA-L contributes to ER stress-induced adiponectin downregulation. To test this, we examined UPR, which is induced by ER stress, in scramble and three independent DsbA-L-suppressed 3T3-L1 cells. In agreement with our

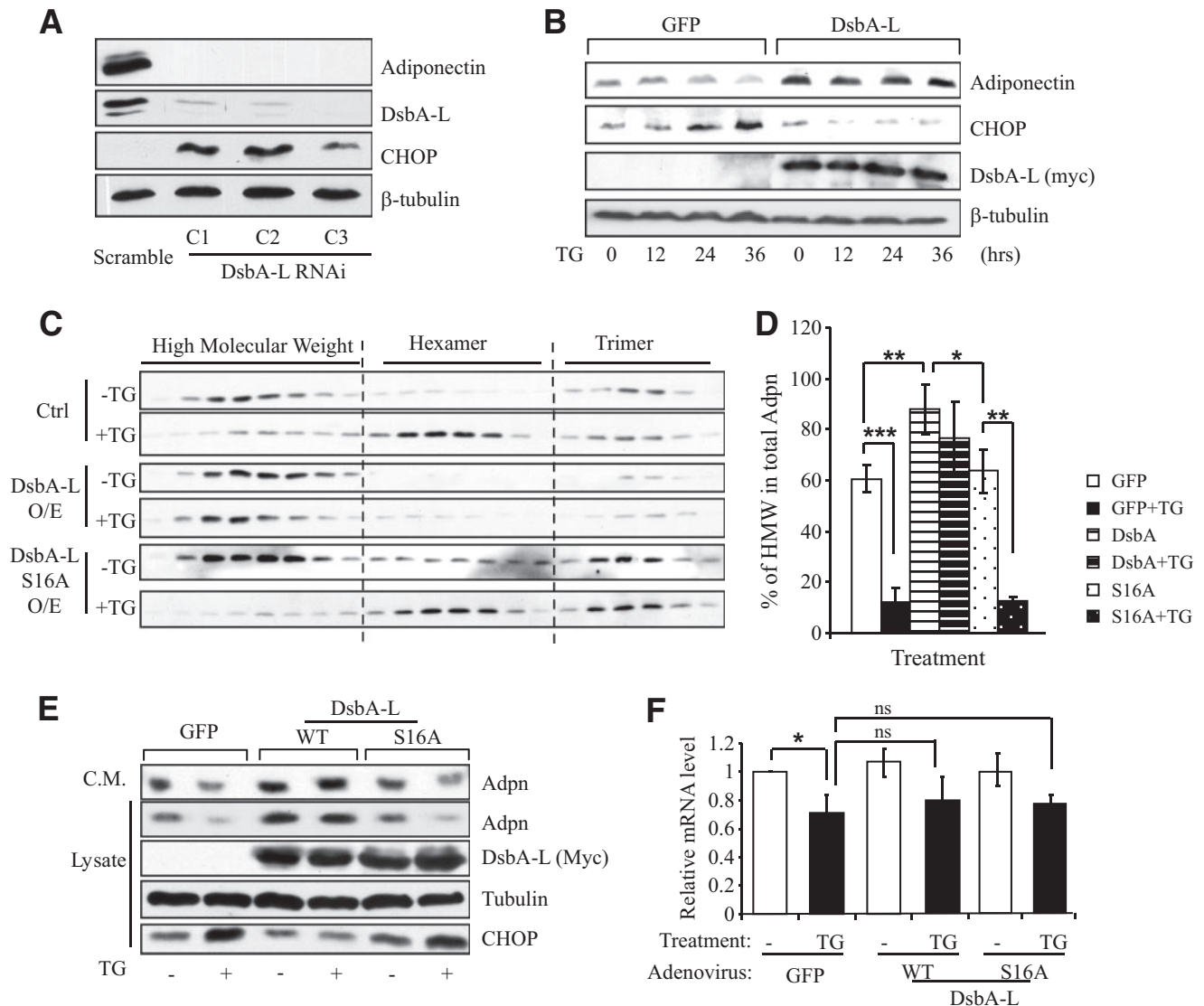


**FIG. 1.** Suppression of ER stress improves adiponectin expression in *db/db* mice. **A:** The expression levels of adiponectin (Adpn), DsbA-L, and CHOP in white adipose tissue (WAT) and the serum adiponectin levels from saline- or TUDCA-treated lean or *db/db* mice were determined by Western blot with specific antibodies as indicated.  $\beta$ -actin or immunoglobulin (IgG) was used as a tissue or serum loading control, respectively. The relative protein levels of adiponectin in WAT (**B**), or in serum (**C**), and DsbA-L (**D**) were quantified. Data represent mean  $\pm$  SEM.  $**P < 0.01$ ,  $***P < 0.001$ ;  $n = 10$  for the saline control mice, and  $n = 8$  per treatment group for the *db/db* mice. **E:** Differentiated 3T3-L1 adipocytes were pretreated with or without TUDCA (1 mmol/l) for 1 h followed with TG (0.01  $\mu$ mol/l) cotreatment for 24 h. The cells were lysed, and the expression levels of adiponectin, DsbA-L, CHOP, TNF $\alpha$ , resistin, and  $\beta$ -tubulin were determined by Western blot analysis with specific antibodies as indicated. Adiponectin levels in conditioned medium were determined with an antiadiponectin antibody. **F:** The mRNA levels of adiponectin were determined by quantitative reverse transcription PCR. The protein levels of adiponectin as shown in (**E**) were quantified (**G**).  $N = 3$ ,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ; ns, no statistical difference; ctrl, control.

previous findings (10), the cellular levels of adiponectin were greatly suppressed in the DsbA-L-suppressed 3T3-L1 adipocytes (Fig. 2A, top panel). Interestingly, UPR was markedly increased in the DsbA-L-suppressed cells, as

demonstrated by increased expression of the UPR marker CHOP (Fig. 2A, third panel). Taken together with our previous finding that suppressing DsbA-L expression in 3T3-L1 adipocytes greatly reduced the protein, but not the





**FIG. 2.** DsbA-L suppresses ER stress and improves adiponectin expression and multimerization. **A:** The expression levels of adiponectin, DsbA-L, and CHOP in DsbA-L-suppressed or scramble control 3T3-L1 adipocytes were determined by Western blot with specific antibodies.  $\beta$ -tubulin was used as a loading control. The data represent three independent experiments with similar results. **B:** The 3T3-L1-CAR adipocytes were infected with adenovirus encoding DsbA-L or GFP for 24 h. The cells were then treated with or without TG (0.01  $\mu\text{mol/l}$ ) for various times as indicated. The expression levels of adiponectin, CHOP, and myc-DsbA-L in cell lysates were determined by Western blot with specific antibodies as indicated.  $\beta$ -tubulin was used as a loading control. The data represent three independent experiments with similar results. **C:** The 3T3-L1-CAR adipocytes were infected with adenovirus encoding GFP, DsbA-L, or the S16A mutant of DsbA-L for 24 h. The cells were then treated with or without TG (0.01  $\mu\text{mol/l}$ ) for 24 h as indicated. Adiponectin multimers in cell lysates were separated by gel filtration and determined by Western blot with an antiadiponectin antibody. The percentages of HMW form in total adiponectin (**D**) were quantified ( $n = 3$ ,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ). **E:** The 3T3-L1-CAR adipocytes were infected with adenoviruses encoding myc-tagged wild-type (WT) or the S16A mutant of DsbA-L for 24 h. The cells were then treated with or without TG (0.01  $\mu\text{mol/l}$ ) for 24 h. The adiponectin levels in conditioned medium (C.M.) and the expression levels of adiponectin, myc-DsbA-L or DsbA-L<sup>S16A</sup>, and CHOP were determined by Western blot with specific antibodies as indicated.  $\beta$ -tubulin was used as a loading control. **F:** The mRNA levels of adiponectin were determined by quantitative reverse transcription PCR.  $N = 3$ ,  $*P < 0.05$ ; ns, no statistical difference.

mRNA, levels of adiponectin (10), these results suggest that downregulation of DsbA-L may lead to accumulation of incorrectly folded proteins that in turn induces UPR. Consistent with this, overexpression of DsbA-L suppressed CHOP expression and enhanced resistance to ER stress-induced adiponectin downregulation in 3T3-L1 CAR adipocytes (Fig. 2B).

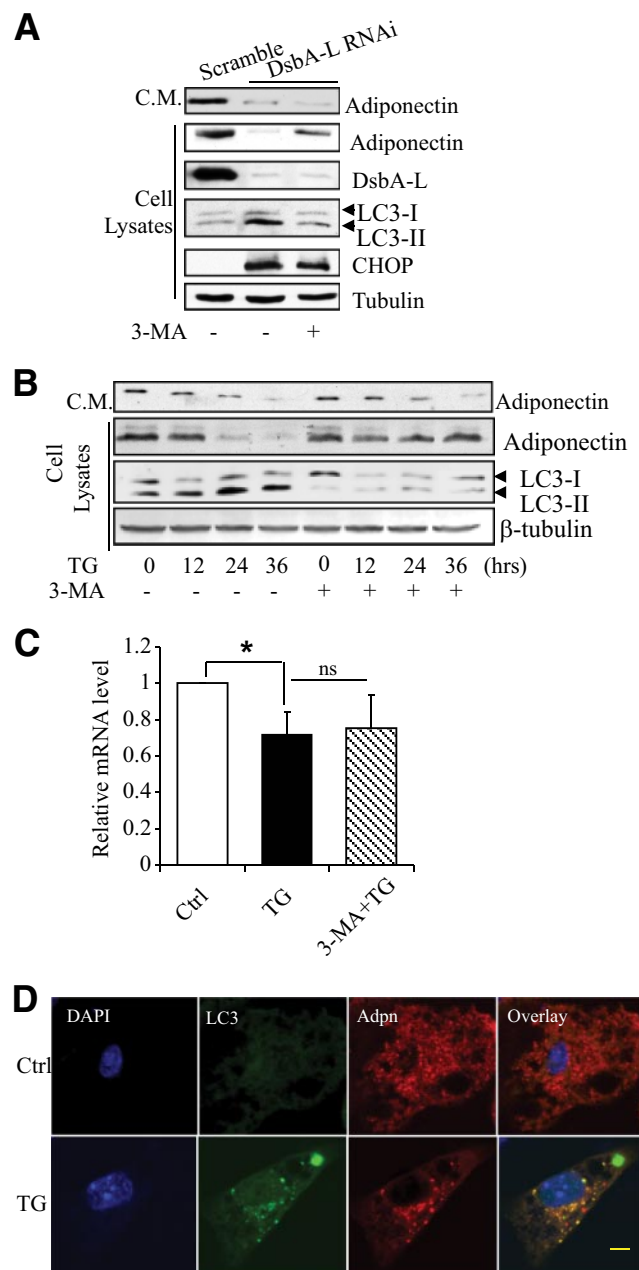
To determine the role of the DsbA-L in ER stress-induced dysregulation of adiponectin multimerization, we studied adiponectin multimer distribution by gel filtration in 3T3-L1-CAR adipocytes. To better compare the effects of TG on the relative distributions of various adiponectin multimers, equal amounts of adiponectin from TG-treated

and nontreated 3T3-L1-CAR cells were analyzed by Western blot. In 3T3-L1-CAR adipocytes overexpressing GFP control, the majority of the adiponectin multimers is the HMW form (Fig. 2C, top panel, and D). Induction of ER stress by TG treatment led to marked reduction of the HMW form of adiponectin, which was alleviated by overexpression of wild-type but not the S16A mutant of DsbA-L (Fig. 2C and D). Overexpression of the S16A mutant of DsbA-L failed to protect ER stress-induced downregulation of cellular and secreted adiponectin (Fig. 2E). Since the DsbA-L<sup>S16A</sup> mutant is unable to bind to adiponectin (10), these findings suggest that the interaction with adiponectin is critical for the protective effect of DsbA-L.

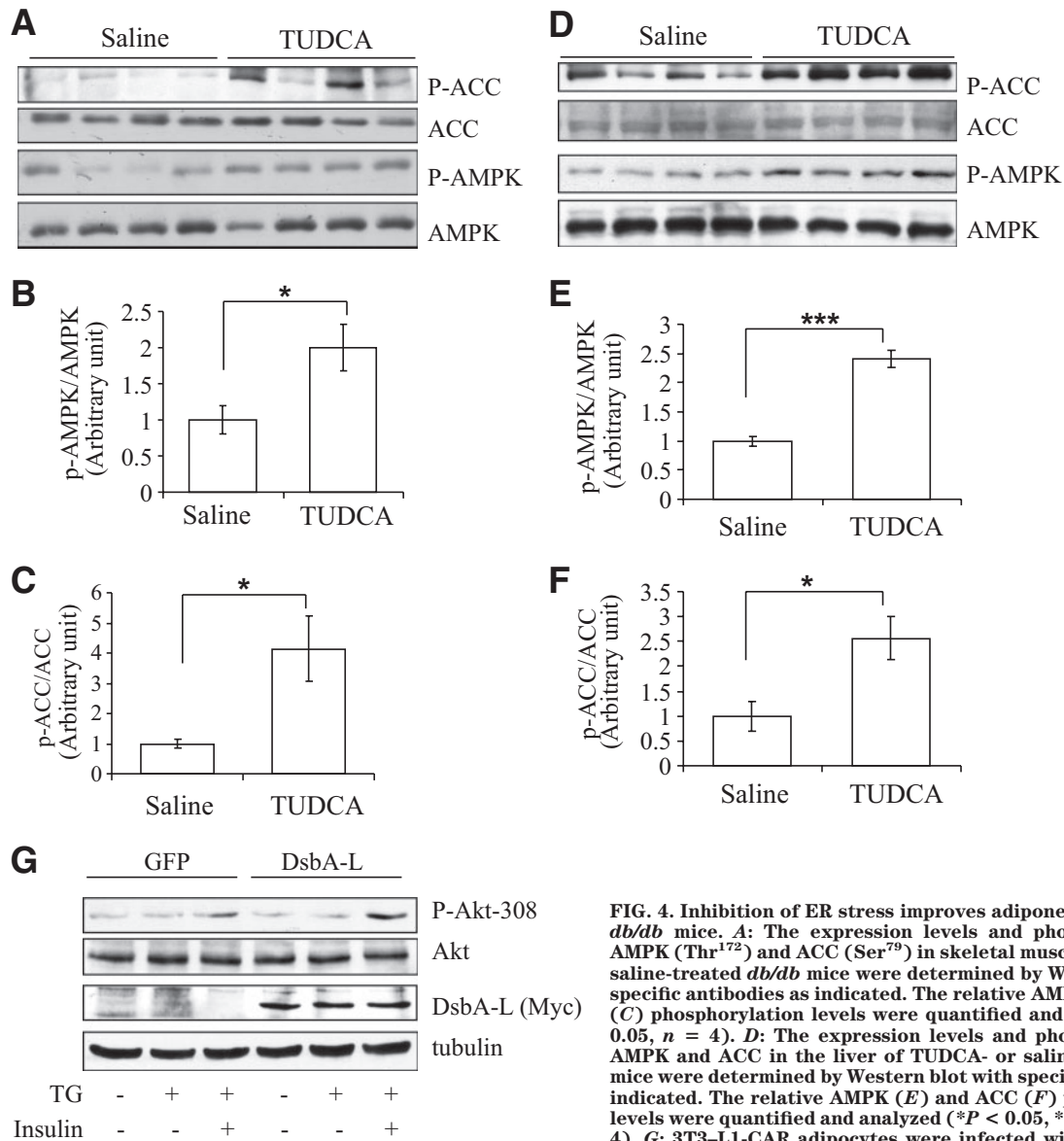
Our results also demonstrated that overexpression of either wild-type or the S16A mutant of DsbA-L had no significant protecting effect on the mRNA levels of adiponectin (Fig. 2F). On the other hand, the protein levels of adiponectin were significantly increased in cells overexpressing wild-type DsbA-L (Fig. 2E). Taken together, these results provide further evidence that the rescuing effect of DsbA-L on cellular levels of adiponectin is mainly at the post-translational level.

**Adiponectin is downregulated in DsbA-L-suppressed cells via autophagy-dependent degradation.** We recently found that autophagy plays a critical role in ER stress-induced insulin receptor downregulation (18). Since ER stress is induced in DsbA-L-suppressed cells (Fig. 2A), we asked whether autophagy is involved in the downregulation of adiponectin expression in these cells. The levels of LC3-II, an autophagy marker protein, were noticeably increased in the DsbA-L-suppressed 3T3-L1 adipocytes compared with the scramble control cells (Fig. 3A). Pretreating the DsbA-L-suppressed adipocytes with the autophagy inhibitor 3-MA, but not the proteasome inhibitor MG-132, greatly suppressed LC3 expression and significantly blocked DsbA-L deficiency-induced adiponectin protein downregulation (Fig. 3A and data not shown). A rescuing effect of 3MA on the total levels of adiponectin was also observed in TG-treated 3T3-L1 adipocytes (Fig. 3B). However, despite elevated cellular adiponectin levels, adiponectin secretion was not significantly increased by the 3MA treatment (Fig. 3A and B, top panel). One possible explanation for these results is that most of the rescuing adiponectin molecules were not correctly folded in the DsbA-L-deficient cells and thus unable to pass the ER-Golgi quality control system for secretion. The 3-MA treatment had no significant rescuing effect on adiponectin mRNA levels (Fig. 3C), consistent with an inhibitory role of 3MA in autophagy-induced protein degradation. Immunofluorescence studies revealed that ER stress promoted adiponectin translocation to the autophagosome, which is evident by increased colocalization of the adiponectin signal with the punctate LC3 staining (Fig. 3D, bottom panel). No major punctate LC3 fluorescent autophagosome signal could be detected in the control cells (Fig. 3D, top panels). These results provide further evidence on the involvement of an autophagy-dependent pathway in ER stress-induced adiponectin degradation.

**Alleviating ER stress improves AMPK signaling and insulin sensitivity in *db/db* mice.** Adiponectin activates AMPK, which in turn phosphorylates and inactivates acetyl-CoA carboxylase (ACC) to regulate fatty acid oxidation (19). Based on the finding that alleviating ER stress increased adiponectin expression levels and secretion in *db/db* mice (Fig. 1), we examined the effect of TUDCA on AMPK and ACC phosphorylation. We found that AMPK activation, as indicated by Thr<sup>172</sup> phosphorylation, was significantly increased in the skeletal muscle of the TUDCA-treated *db/db* mice (Fig. 4A and B). Suppressing ER stress by TUDCA also led to a great increase in ACC phosphorylation at the AMPK-mediated phosphorylation site (Ser<sup>79</sup>) (Fig. 4A and C). A similar increase in AMPK activation was observed in the liver of TUDCA treated *db/db* mice (Fig. 4D–F). To determine whether DsbA-L has an effect on insulin signaling, we infected 3T3-L1-CAR adipocytes with adenoviruses encoding GFP or GFP plus DsbA-L. We found that overexpression of DsbA-L increased the cellular levels and secretion of adiponectin and enhanced resistance to TG-induced ER stress (Fig.



**FIG. 3.** ER stress or DsbA-L knockdown suppresses adiponectin levels by inducing autophagy-dependent degradation. **A:** DsbA-L-suppressed or scramble control 3T3-L1 adipocytes were treated with 3-MA (5  $\mu$ M) or vehicle for 24 h as indicated. The protein levels of adiponectin, DsbA-L, and LC3 in cell lysates were determined by Western blot using specific antibodies as indicated.  $\beta$ -tubulin was used as a loading control. Adiponectin levels in conditioned medium (C.M.) were determined with an antiadiponectin antibody. The data represent three independent experiments with similar results. **B:** The 3T3-L1 adipocytes were pretreated with (+) or without (-) 3-MA (5  $\mu$ M) for 1 h, followed by TG (0.01  $\mu$ M) for different times as indicated. The protein levels of adiponectin, LC3, and  $\beta$ -tubulin were determined by Western blot analysis with specific antibodies as indicated. Adiponectin levels in conditioned medium (C.M.) were determined with an antiadiponectin antibody. The mRNA levels of adiponectin (**C**) were determined by quantitative reverse transcription PCR;  $N = 3$ ,  $*P < 0.05$ ; ns, no statistical difference. **D:** Differentiated 3T3-L1 adipocytes were treated with TG (0.01  $\mu$ M) or vehicle for 36 h, and then fixed. The cellular localization of adiponectin (Adpn, red) was determined by immunofluorescence-staining with a specific antibody to the protein. The cellular localization of GFP-LC3 was observed by green fluorescent protein fluorescence. In each experiment, more than 100 cells from each group were checked, and  $\sim 70\%$  of the cells showed a similar pattern. The data represent three independent experiments with similar results. (A high-quality digital representation of this figure is available in the online issue.)



**FIG. 4.** Inhibition of ER stress improves adiponectin signaling in *db/db* mice. **A:** The expression levels and phosphorylation of AMPK (Thr<sup>172</sup>) and ACC (Ser<sup>79</sup>) in skeletal muscle of TUDCA- or saline-treated *db/db* mice were determined by Western blot with specific antibodies as indicated. The relative AMPK (**B**) and ACC (**C**) phosphorylation levels were quantified and analyzed (\* $P < 0.05$ ,  $n = 4$ ). **D:** The expression levels and phosphorylation of AMPK and ACC in the liver of TUDCA- or saline-treated *db/db* mice were determined by Western blot with specific antibodies as indicated. The relative AMPK (**E**) and ACC (**F**) phosphorylation levels were quantified and analyzed (\* $P < 0.05$ , \*\*\* $P < 0.001$ ,  $n = 4$ ). **G:** 3T3-L1-CAR adipocytes were infected with adenoviruses encoding GFP or GFP plus myc-DsbA-L for 24 h. The cells were

then pretreated with or without TG (0.01  $\mu\text{mol/l}$ ) for 24 h as indicated, followed with or without 10 nmol/l insulin stimulation for 5 min. The expression levels of myc-tagged DsbA-L, Akt, and phosphor-Akt (Thr<sup>308</sup>) in cell lysates were determined by Western blot with specific antibodies as indicated.  $\beta$ -tubulin was used as a loading control. The experiment represents three independent experiments with similar results.

2B) and insulin-stimulated Akt phosphorylation (Fig. 4G). Consistent with the result that suppressing ER stress increased insulin signaling, insulin tolerance test experiments showed that TUDCA treatment increased insulin sensitivity in *db/db* mice (online supplemental Fig. S3A). TUDCA treatment did not stimulate AMPK activation in cultured C2C12 myotubes and murine hepatocytes (online supplemental Fig. S4), suggesting that the increased AMPK/ACC phosphorylation and insulin sensitivity in vivo is not caused by a direct stimulatory effect of this chemical chaperone.

## DISCUSSION

It is well documented that the cellular and serum levels of adiponectin are negatively correlated with obesity. However, the precise underlying mechanisms remain unclear. Obesity leads to a low-grade chronic inflammatory state accompanied by increased production of proinflammatory cytokines such as TNF- $\alpha$  and interleukin (IL)-6, which

have been shown to reduce adiponectin gene expression (15,20,21). Obesity also results in a hypoxic microenvironment (22,23), leading to increased ER stress and attenuated adiponectin promoter activity (15). In the present study, we show that inducing ER stress is sufficient to decrease the protein levels of adiponectin in 3T3-L1 adipocytes (Fig. 2), suggesting a causative role of ER stress in downregulating the cellular levels of adiponectin. Consistent with this view, inhibiting ER stress by the chemical chaperone TUDCA partially restores adiponectin levels in both *db/db* mice (Fig. 1) and HFD-induced obese mice (online supplemental Fig. S2). Our results are in agreement with an earlier finding that treating the *db/db* mice with peroxisome proliferator-activated receptor (PPAR) $\alpha/\gamma$  dual agonist macelignan, which reduces ER stress, increases adiponectin expression in adipose tissue of *db/db* mice (24).

Obesity reduces the cellular levels of PPAR $\gamma$ , which has been shown to downregulate adiponectin transcription



(25). However, a recent study showed that activation of PPAR $\gamma$  by pioglitazone treatment increased plasma levels of adiponectin in human subjects, but had no effect on adiponectin expression in adipose tissue (26), suggesting that the primary effect of PPAR $\gamma$  may be to promote adiponectin assembly and secretion, rather than transcription. Consistent with this notion, treating adipocytes with PPAR $\gamma$  agonists increased the synthesis and secretion of HMW adiponectin without affecting mRNA expression and protein synthesis of this adipokine (5,27). A likely mechanism by which PPAR $\gamma$  enhances adiponectin cellular levels and secretion is to increase the expression levels of ER chaperones such as Ero1- $\alpha$ , which have been shown to promote adiponectin assembly and secretion in mature adipocytes and in mice (5,7). Interestingly, we recently found that treating 3T3-L1 adipocytes with rosiglitazone increases the expression levels of DsbA-L (10). Since suppressing DsbA-L leads to ER stress and adiponectin downregulation (Fig. 2A), these results suggest that DsbA-L may enhance adiponectin stability and secretion by enhancing ER function.

In obesity, the cellular levels of DsbA-L are reduced (10), which may contribute to increased adiponectin misfolding in the ER and blockage of this adipokine to form HMW multimers necessary for secretion. The obesity-induced accumulation of misfolded molecules in the ER may result in ER stress and UPR, which in turn lead to degradation of incorrectly folded proteins by either the proteasome- or autophagy-associated protein degradation pathway (28,29). We found that treating the ER-stressed 3T3-L1 cells with 3MA, but not MG132, greatly rescued adiponectin levels (Fig. 3), suggesting that ER stress-induced adiponectin downregulation is mediated by an autophagy-dependent mechanism. Our data showed that the expression levels of endogenous DsbA-L were reduced in TG-treated cells (Fig. 1E), suggesting that DsbA-L reduction is a consequence of ER stress. This result is consistent with our previous finding that DsbA-L expression levels are negatively correlated with obesity in both obese human subjects and animal models of obesity (10). Interestingly, we also found that ER stress is increased in DsbA-L-suppressed cells (Figs. 2A and 3A). One possible explanation for these seemingly contradictory results is that DsbA-L plays a critical role in the maintenance of cell homeostasis, probably functioning as a chaperone to facilitate correct folding and assembly of adiponectin and potentially other macromolecules in the ER, where transmembrane and secreted proteins are synthesized, assembled, and/or modified. Thus, reducing its expression levels may lead to overloading of incorrectly-folded proteins, resulting in ER stress and subsequent activation of an autophagy-dependent protein-degradation pathway. Thus, the negative effect of obesity on adiponectin levels may be twofold: obesity leads to overloading of misfolded adiponectin in the ER, which may induce ER stress-induced degradation. Obesity also downregulates DsbA-L, thus accelerating adiponectin misfolding and degradation by diminishing the protective chaperone activity of DsbA-L. Consistent with this model, overexpression of DsbA-L promotes the assembly of the HMW form of adiponectin and prevents ER stress-induced adiponectin downregulation (Fig. 2B and C).

In conclusion, we have presented evidence showing that inducing ER stress is sufficient to cause adiponectin degradation in 3T3-L1 adipocytes. In addition, we show that DsbA-L has a protective role in ER stress-induced

adiponectin downregulation. Since DsbA-L levels are reduced in obese human subjects and animal models of obesity (10), reduction of DsbA-L could be a mechanism by which obesity downregulates adiponectin levels and secretion, contributing to increased insulin resistance. We previously showed that the cellular levels of DsbA-L are stimulated by the PPAR $\gamma$  agonist rosiglitazone (10), suggesting that increasing the expression levels of this protein could be a promising approach to increase adiponectin multimerization and insulin sensitivity.

#### ACKNOWLEDGMENTS

This work was supported by National Institutes of Health RO1 grants DK -76902 (to F.L.) and DK-69930 (to L.Q.D.).

No potential conflicts of interest relevant to this article were reported.

L.Z. designed the research, performed research, analyzed data, and wrote the manuscript. M.L., J.Z., and H.C. performed research. L.Q.D. analyzed data. F.L. designed the research, analyzed data, and wrote the manuscript.

The authors are grateful to Derong Hu, Department of Pharmacology, University of Texas Health Science Center at San Antonio, for excellent technical assistance.

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