DsbA-L Alleviates Endoplasmic Reticulum Stress-induced Adiponectin Down-regulation

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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 DsbA-L, a recently identified adiponectin interactive protein that promotes adiponectin multimerization and stability (Liu et al. (2008) Proc Natl Acad Sci U S A. 105, 18302-18307), in obesity-induced endoplasmic reticulum (ER) stress and adiponectin down-regulation.

Research design and methods—Tauroursodeoxycholic acid (TUDCA), a chemical chaperone that alleviates ER stress, was used to study the mechanism underlying obesity-induced adiponectin down-regulation 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 down-regulation 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 down-regulate 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 down-regulation. In addition, DsbA-L facilitates adiponectin folding and assembly and provides a protective effect against ER stress-mediated adiponectin down-regulation in obesity.

Adiponectin 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 (ER) into primarily 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-Lα, 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 a number of 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 secretory 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 down-regulation remain to be established.

We have recently identified an adiponectin interacting protein named disulfide-bond A oxidoreductase-like protein or 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 db/db mice and diet-induced obese mice. In addition, we demonstrate that inducing ER stress is sufficient to down-regulate 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 down-regulation of adiponectin and increasing the cellular levels of DsbA-L improves adiponectin assembly and stability by suppressing the negative effect of ER stress.

**RESEARCH DESIGNS 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 Inc.), tauroursodeoxycholic acid (TUDCA) (580549, Calbiochem, Gibbstown, NJ), and G418 sulfate (30-234-CR, Cellgro, Manassas, VA). Polyclonal anti-adiponectin and DsbA-L antibodies were generated as described before (10). Antibodies against β-actin, AMPK, phosphor-Thr172-AMPK, Acetyl-CoA carboxylase, phosphor-Ser79-Acetyl-CoA carboxylase, and anti-LC3 were from Cell Signaling Technology (Danvers, MA). Other antibodies used were: monoclonal anti-adiponectin (MAB3608, Chemicon International Inc.), anti-β-tubulin 2.1 (T4026, Sigma-Aldrich Inc.), 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). 3T3-L1 cells stably expressing EGFP-LC3 were generated by transfection of cells with the pEGFP-LC3 plasmid, a generous gift of Dr. Jean X. Jiang (UTHSCSA), and selection with 400 μg/ml of
G418. Independent clones were selected by limited dilution. 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 22–24°C on a 12-hr light/12-hr dark cycle with the lights on at 8:00 am. 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 i.p. injected with TUDCA (250 mg/kg) or equal volumes of vehicle (saline) twice daily (8 am and 8 pm) for 26 days. Mice were then sacrificed and mouse tissues were isolated according to the procedure as described in our recent studies (13). For high fat diet (HFD) experiments, 6–8 week old C57BL/6 mice (10 mice/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 HFD feeding period, the mice were i.p. injected with TUDCA (250 mg/kg) or equal volumes of vehicle (saline) twice daily (8 am and 8 pm). Mice were sacrificed and mouse tissues were isolated according to the procedure as 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 (ITT).** The saline- and TUDCA-treated male db/db mice and lean control mice (8–10 mice/group) were fasted for 6 hrs 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 by using an automatic glucometer (Rightest GM300; Bionime Corp, San Diego, CA).

**Determination of adiponectin multimers.** Adiponectin multimers were determined by gel filtration FPLC with a Superdex™ 200 10/30 column (GE Healthcare Bio-Sciences Corp., 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 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 (QRT-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). cDNA samples were amplified in duplicate in 96 microtiter plates (Applied
Biosystems). Each PCR reaction (20 µl total volume) contained: 10 µl of SYBR Green PCR Master Mix (Applied Biosystems), 5pmols of each primer and 1 µg 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 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’-TCCACCACCTGTTGCTGTA-3’.

Real-time PCR reactions were carried out in an ABI PRISM 7700 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 was performed using analysis of variance (ANOVA). Statistical significance was set at $P$ values of *$<0.05$, **$<0.01$, and ***$<0.001$.

**RESULTS**

**Reduction of ER stress increases adiponectin levels in obese mice and in cells.** Obesity induces ER stress, the latter is correlated with reduced adiponectin levels (15). To determine if ER stress is involved in down-regulating 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 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 to 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 (Figs. 1A – 1D). TUDCA treatment had no significant effect on the food intake (data not shown and (16)) and body weight of the db/db mice (Fig. S1A in the online appendix available at http://diabetes.diabetesjournals.org), 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 (Fig. S2). Taken together, these results suggest that ER stress may play a key role in obesity-induced adiponectin down-regulation.

**DsbA-L prevents ER stress-induced adiponectin down-regulation in 3T3-L1 adipocytes.** To determine if 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 pre-treating the cells with TUDCA (Fig. 1E). While inducing
ER stress by TG led to an approximately 25% reduction in the mRNA levels of adiponectin, treating 3T3-L1 adipocytes with TUDCA had no significant rescuing effect on the TG-induced down-regulation 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 down-regulation of adiponectin was significantly protected by treating the cells with TUDCA (Fig. 1G), suggesting 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, TNFα 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 down-regulation. 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 previous findings (10), the cellular levels of adiponectin were greatly suppressed in the DsbA-L-suppressed 3T3-L1 adipocytes (Fig 2A, the 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, the 3rd panel). Taken together with our previous findings that suppressing DsbA-L expression in 3T3-L1 adipocytes greatly reduced the protein but not the mRNA levels of adiponectin (10), these results suggest that down-regulation 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 down-regulation 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 none-treated 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, the top panel and Fig. 2D). 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 (Figs. 2C and 2D). Overexpression of the S16A mutant of DsbA-L failed to protect ER stress-induced down-regulation of cellular and secreted adiponectin (Fig. 2E). Since the DsbA-L S16A 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 down-regulated in DsbA-L-suppressed cells via autophagy-dependent degradation. We have recently found that autophagy plays a critical role in ER stress-induced insulin receptor down-regulation (18). Since ER stress is induced in DsbA-L-suppressed cells (Fig. 2A), we asked whether autophagy is involved in the down-regulation 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 to the scramble control cells (Fig. 3A). Pre-treating 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 down-regulation (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 (Figs. 3A and 3B, 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. 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 Thr172 phosphorylation, was significantly increased in skeletal muscle of the TUDCA-treated db/db mice (Figs. 4A and B). Suppressing ER stress by TUDCA also led to a great increase in ACC phosphorylation at the AMPK-mediated phosphorylation site (Ser79) (Figs 4A and C). A similar increase in AMPK activation was observed in the liver of TUDCA treated db/db mice (Figs 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 2B) and insulin-stimulated Akt phosphorylation (Fig 4G). Consistent with the result that suppressing ER stress increased insulin signaling, ITT experiments showed that TUDCA treatment increased insulin sensitivity in db/db mice (Fig S3A). TUDCA treatment did not stimulate AMPK activation in cultured C2C12 myotubes and murine hepatocytes (Fig. S4), suggesting that the increased AMPK/ACC phosphorylation and insulin sensitivity in vivo is not due to 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 with increased production of proinflammatory cytokines such as TNF-α and 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 down-regulating the cellular levels of adiponectin. Consistent with this view, inhibiting ER stress by the chemical chaperone TUDCA partially restores adiponectin levels in db/db mice (Fig. 1) and HFD-induced obese mice (Fig. S2). Our results are in agreement with an earlier finding that treating the db/db mice with PPARα/γ dual agonist macelignan, which reduces ER stress, increases adiponectin expression in adipose tissue of db/db mice (24).

Obesity reduces the cellular levels of PPARγ, which has been shown to down-regulate adiponectin transcription (25). However, a recent study showed that activation of PPARγ 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 PPARγ may be to promote adiponectin assembly and secretion, rather than transcription. Consistent with this notion, treating adipocytes with PPARγ 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 PPARγ enhances adiponectin cellular levels and secretion is to increase the expression levels of ER chaperones such as Ero1-Lα, 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 down-regulation (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 mis-folding in the ER
and blockage of this adipokine to form HMW multimers necessary for secretion. The obesity-induced accumulation of mis-folded 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 down-regulation is mediated by an autophagy-dependent mechanism. Our data showed that the expression levels of endogenous DsbA-L were reduced in TG-treated cells (Figs. 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 two-fold: obesity leads to overloading of misfolded adiponectin in the ER, which may induce ER-stress-induced degradation. Obesity also down-regulates 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 down-regulation (Figs. 2B and 2C).

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 down-regulation. 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 down-regulates adiponectin levels and secretion, contributing to increased insulin resistance. We previously showed that the cellular levels of DsbA-L are stimulated by the PPARγ agonist rosiglutazone (10), suggesting that increasing the expression levels of this protein could be a promising approach to increase adiponectin multimerization and insulin sensitivity.

**Author contributions:** L. Z. and F. L. designed research; L. Z., M. L. J. Z. and H. C. performed research; L.Z., L.Q.D and F.L. analyzed data; and L.Z. and F.L. wrote the paper.

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REFERENCES


Figure legends

Figure 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. β-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 ± SEM. **P <0.01, ***P<0.001; n=10 for the saline control mice and n=8/treatment group for the db/db mice. (E) Differentiated 3T3-L1 adipocytes were pre-treated with or without TUDCA (1 mM) for 1 hr followed with TG (0.01 μM) co-treatment for 24 hrs. The cells were lysed and the expression levels of adiponectin, DsbA-L, CHOP, TNFα, resistin, and β-tubulin were determined by Western blot with specific antibodies as indicated. Adiponectin levels in conditioned medium were determined with an anti-adiponectin 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.

Figure 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. β-tubulin was used as a loading control. The data represents 3 independent experiments with similar results. (B) 3T3-L1-CAR adipocytes were infected with adenovirus encoding DsbA-L or GFP for 24 hrs. The cells were then treated with or without TG (0.01μM) for various times as indicated. The expression levels of adiponectin, CHOP, myc-DsbA-L in cell lysates were determined by Western blot with specific antibodies as indicated. β-tubulin was used as loading control. The data represents 3 independent experiments with similar results. (C) 3T3-L1-CAR adipocytes were infected with adenovirus encoding GFP, DsbA-L, or the S16A mutant of DsbA-L for 24 hrs. The cells were then treated with or without TG (0.01μM) for 24 hours as indicated. Adiponectin multimers in cell lysates were separated by gel filtration and determined by Western blot with an anti-adiponectin antibody. The percentages of high molecular weight (HMW) form in total adiponectin (D) were quantified. (n=3, *p<0.05, **p<0.01, ***p<0.001) (E) 3T3-L1-CAR adipocytes were infected with adenoviruses encoding myc-tagged wild-type (WT) or the S16A mutant of DsbA-L for 24 hrs. The cells were then treated with or without TG (0.01μM) for 24 hours as indicated. Adiponectin multimers in cell lysates were separated by gel filtration and determined by Western blot with an anti-adiponectin antibody. The percentages of high molecular weight (HMW) form in total adiponectin (D) were quantified. (n=3, *p<0.05, **p<0.01, ***p<0.001) (E) 3T3-L1-CAR adipocytes were infected with adenoviruses encoding myc-tagged wild-type (WT) or the S16A mutant of DsbA-L for 24 hrs. The cells were then treated with or without TG (0.01μM) for 24 hours. The adiponectin levels in conditioned medium (C.M.) and the expression levels of adiponectin, myc-DsbA-L or DsbA-LS16A, and CHOP were determined by western blot with specific antibodies as indicated. β-tubulin was used as loading control. (F) The mRNA levels of adiponectin were determined by quantitative reverse transcription PCR. N=3, *p<0.05, ns: no statistical difference.
Figure 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 μM) or vehicle for 24 hrs as indicated. The protein levels of adiponectin, DsbA-L, LC3 in cell lysates were determined by Western blot using specific antibodies as indicated. β-tubulin was used as a loading control. Adiponectin levels in conditioned medium (C.M.) were determined with an anti-adiponectin antibody. The data represents 3 independent experiments with similar results. (B) 3T3-L1 adipocytes were pre-treated with (+) or without (-) 3-MA (5 μM) for 1 hr, followed by TG (0.01 μM) for different time as indicated. The protein levels of adiponectin, LC3 and β-tubulin were determined by Western blot with specific antibodies as indicated. Adiponectin levels in conditioned medium (C.M.) were determined with an anti-adiponectin 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 thapsigargin (TG, 0.01μM) or vehicle for 36 hrs 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 GFP fluorescence. In each experiment, over 100 cells from each group were checked and approximately 70% of the cells showed a similar pattern. The data represents 3 independent experiments with similar results.

Figure 4. Inhibition of ER stress improves adiponectin signaling in db/db mice.

(A) The expression levels and phosphorylation of AMPK (Thr172) and ACC (Ser79) 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 hrs. The cells were then pre-treated with or without TG (0.01μM) for 24 hrs as indicated, followed with or without 10 nM insulin stimulation for 5 minutes. The expression levels of myc-tagged DsbA-L, Akt and phosphor-Akt (Thr308) in cell lysates were determined by Western blot with specific antibodies as indicated. β-tubulin was used as loading control. The experiment represents 3 independent experiments with similar results.
Figure 1

A

Lean; Saline

TUDCA

Saline

WAT

Adpn

DsbA-I

CHOP

β-actin

Serum

Adpn

IgG

B

Cellular adiponectin level (Arbitrary unit)

Lean

db/db

+TUDCA

db/db

C

Circulating adiponectin level (Arbitrary unit)

Lean

db/db

+TUDCA

db/db

D

Arbitrary DsbA-I level

Lean

db/db

+TUDCA

db/db

E

Cell Lysates

Adpn

DsbA-I

CHOP

Tubulin

Resistin

TNF-α

Medium

Adpn

TG

+ +

TUDCA

- + +

F

Relative AdipmRNA level

Ctrl

TG

TUDCA-TG

G

Relative Adip protein level

Ctrl

TG

TUDCA-TG
Figure 4

A

Saline  TUDCA

P-ACC  ACC  P-AMPK  AMPK

B

\[
\begin{array}{c}
\text{Saline}  \quad \text{TUDCA} \\
\hline
\text{p-AMPK/AMPK}  \\
\text{(Arbitrary unit)} \\
\end{array}
\]

C

\[
\begin{array}{c}
\text{Saline}  \quad \text{TUDCA} \\
\hline
\text{p-ACC/ACC}  \\
\text{(Arbitrary unit)} \\
\end{array}
\]

D

Saline  TUDCA

P-ACC  ACC  P-AMPK  AMPK

E

\[
\begin{array}{c}
\text{Saline}  \quad \text{TUDCA} \\
\hline
\text{p-AMPK/AMPK}  \\
\text{(Arbitrary unit)} \\
\end{array}
\]

F

\[
\begin{array}{c}
\text{Saline}  \quad \text{TUDCA} \\
\hline
\text{p-ACC/ACC}  \\
\text{(Arbitrary unit)} \\
\end{array}
\]

G

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