Fat-Specific DsbA-L Overexpression Promotes Adiponectin Multimerization and Protects Mice From Diet-Induced Obesity and Insulin Resistance

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Adiponectin is the only known adipokine whose levels are downregulated in obesity (8). Pharmacological studies have demonstrated that acutely enhancing the globular form of adiponectin in mice significantly increased fatty acid oxidation and reduced body weight (16,17). Transgenic overexpression of full-length adiponectin or the globular form of adiponectin has been shown to increase energy expenditure, insulin sensitivity, and fatty acid oxidation (16–20). Taken together, these results suggest that increasing serum adiponectin levels might be an attractive therapeutic approach for the treatment of obesity-induced metabolic diseases. However, the serum levels of adiponectin are extremely high, ranging between 1 and 20 μg/mL (21). Such a high concentration, which is at least three orders of magnitude higher than the levels of other adipokines, such as leptin and interleukin-6 (IL-6), results in technical difficulties in the development of adiponectin-based antidiabetic and antiatherogenic strategies.

An important finding in the adiponectin research field is that complex distribution, rather than the total levels of adiponectin, is associated with improved insulin sensitivity in response to thiazolidinedione stimulation in mice and humans (22). Consistent with this finding, the HMW form of adiponectin has been demonstrated as having major biological functions in regulating glucose homeostasis (23–25). In contrast, impairment of adiponectin multimerization affects both secretion and function of this adipokine and is associated with diabetes and hyperadiponectinemia (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.

We have recently identified the disulfide bond A oxidoreductase-like protein (DsbA-L) as a key regulator of adiponectin multimerization in 3T3-L1 cells (26). In addition, we have found that overexpression of DsbA-L, the levels of which are significantly reduced in obese mice and human subjects (26), protected adiponectin from endoplasmic reticulum (ER) stress–induced downregulation in 3T3-L1 cells (27). However, whether overexpression of DsbA-L promotes adiponectin multimerization and improves insulin sensitivity in vivo remains unknown.

In the current study, we show that adipose tissue–specific overexpression of DsbA-L increases adiponectin multimerization and stability in mice. The fat-specific DsbA-L transgenic mice (DshA-L) exhibited enhanced activity and energy expenditure and increased resistance to diet-induced obesity and insulin resistance.
under the curve (AUC) over the basal value of GTT during the time period of 120 min was calculated using the trapezoidal rule and used as an index of glucose tolerance. The incremental area below the basal value of ITT during the time period of 90 min was calculated using the trapezoidal rule and used as an index of insulin tolerance.

**Hyperinsulinemic-euglycemic clamp.** The left carotid artery and right jugular vein of the HFD-fed mice were cannulated by surgery under anesthetized conditions, followed by at least a 5-day recovery period. Insulin sensitivity in the mice was assessed directly with a hyperinsulinemic-euglycemic clamp as described previously (29).

**Energy expenditure.** Before each experiment, the mice were placed in the Multiple Animal Respiratory System (MARS; Sable Systems International, Las Vegas, NV) for at least 12 h to acclimate to a new environment. Oxygen consumption (V\textsubscript{O2}), carbon dioxide production (V\textsubscript{CO2}), and the activity of each animal in live-in cages were measured for 48 h, including two complete light cycles (two for 14 h) and two complete dark cycles (two for 10 h). The respiratory quotient, which provides information on the metabolizable substrates being used, was calculated by the ratio V\textsubscript{CO2}/V\textsubscript{O2}. Activity monitoring was performed simultaneously with metabolic measurements via the MAD-1 Motion/Activity Detector.

**Statistics.** Statistical analysis of the data was performed using ANOVA or Student t test. Statistical significance was set at P values of <0.05 and <0.01.

**RESULTS**

**Generation of fat-specific DsbA-L transgenic mice.** We have recently identified DsbA-L as a critical regulator of adiponectin multimerization in 3T3-L1 adipocytes (26). To determine the functional role of DsbA-L in adiponectin multimerization in vivo, we generated adipose-specific DsbA-L transgenic mice (DsbA-L) using the murine FABP4/aP2 promoter (Fig. 1A). Western blot analysis revealed that the myc-tagged DsbA-L is specifically expressed in fat tissues (Fig. 1B), including white adipose tissue (WAT), brown adipose tissue (BAT), and macrophage, of the DsbA-L transgenic mice with an approximately twofold higher expression compared with that of the endogenous protein (Fig. 1C–E). A similar expression level of DsbA-L was found in an independent transgenic mouse line (data not shown).

**Overexpression of DsbA-L enhances adiponectin multimerization in vivo.** To determine whether overexpression of DsbA-L promotes adiponectin multimerization and stability in vivo, we measured total adiponectin levels and the ratio of each multimer to total adiponectin levels in both adipose tissue and serum. Consistent with our previous finding that DsbA-L promotes adiponectin assembly and stability in 3T3-L1 adipocytes (26), the protein levels of adiponectin and the ratio of the HMW form of this adipokine are increased in adipose tissue (Fig. 2A–D and serum (Fig. 2E–H)) of the DsbA-L mice compared with the control mice. Overexpression of DsbA-L greatly protected mice from HFD-induced downregulation of adiponectin levels (Fig. 2A and B) and its secretion (Fig. 2E and F). Gel filtration studies revealed a significant increase in the HMW form of adiponectin in WAT (Fig. 2C and D) and serum (Fig. 2G and H) of the DsbA-L mice compared with WT littermates under HFD-feeding conditions. Similar results were also observed in another DsbA-L overexpression transgenic mouse line (data not shown). These results provide the first evidence that DsbA-L promotes adiponectin multimerization in vivo.

Overexpression of DsbA-L in mice increased resistance to diet-induced obesity. To determine whether overexpression of DsbA-L has an effect on energy homeostasis, we compared body weight and food intake between the DsbA-L transgenic mice and WT littermates. On normal chow, the DsbA-L mice showed little difference in food intake and body weight compared with WT littermates (data not shown). There was also no significant difference in food intake between DsbA-L and WT littermates fed
mice (data not shown), suggesting that there is no difference in substrate utilization between these mice. Taken together, these results suggest that increased metabolic rate may provide a mechanism by which overexpression of DsbA-L protects mice from diet-induced obesity.

The fDsbA-L mice are resistant to HFD-induced insulin resistance. The fDsbA-L mice displayed increased glucose and insulin tolerance when compared with WT littermates under normal chow diet (Supplementary Fig. 1C and D), but the difference between fDsbA-L and WT mice did not reach statistical significance. Under HFD, the fDsbA-L mice showed a significantly enhanced glucose and insulin tolerance compared with WT littermates (Fig. 4A–D). Hyperglycemic clamp experiments revealed that insulin had a greater suppressive effect on hepatic glucose production in fDsbA-L mice compared with WT mice under the HFD condition (Fig. 4E). The fDsbA-L mice also exhibited a higher insulin-mediated glucose infusion rate (Fig. 4F and G) and lower levels of fasting triglycerides compared with control mice (Supplementary Fig. 2A). In agreement with previous findings that the HMW form of adiponectin has a major insulin-sensitizing effect in the liver (2,25,28,30), AMPK phosphorylation and insulin-stimulated Akt phosphorylation were significantly enhanced in the liver (Fig. 4H and I) and WAT (Supplementary Fig. 2D and C), but not in skeletal muscle (Supplementary Fig. 2D and E), of the fDsbA-L mice compared with WT littermates.

Fat-specific overexpression of DsbA-L protects mice from HFD-induced inflammation and hepatic steatosis. Since liver is the major organ for adiponectin action in vivo (2,25,30), we asked whether fat-specific overexpression of DsbA-L protects mice from HFD-induced inflammation and liver dysfunction. In agreement with the findings of others (31,32), HFD feeding resulted in a large increase in macrophage infiltration into adipose tissue (Fig. 5A and B). The HFD-induced fatty liver was significantly protected in the fDsbA-L mice (Fig. 5C). Overexpression of DsbA-L also greatly reduced HFD-induced macrosteatosis and accumulation of lipid droplets in the liver (Fig. 5D). Consistent with these findings, the expression levels of TNF-α and IL-6 in WAT were significantly reduced in the fDsbA-L mice compared with WT littermates fed with an HFD (Fig. 5E).

Targeted deletion of the adiponectin gene diminishes the beneficial effects of DsbA-L on insulin resistance and hepatic steatosis in mice. To determine whether the beneficial effect of fat-specific overexpression of DsbA-L is mediated by adiponectin multimerization and action, we generated fat-specific DsbA-L transgenic mice in which the adiponectin gene targeted is disrupted (fDsbA-L/Ad−/−) (Fig. 6A). There was little difference in food intake (data not shown), body weight (Fig. 6B and Supplementary Fig. 3A), and fat mass (Fig. 6C) between HFD-fed fDsbA-L/Ad−/− and fDsbA-L mice. In addition, the promoting effect of DsbA-L on activity and energy expenditure was not significantly affected in the fDsbA-L/Ad−/− mice compared with fDsbA-L mice (Supplementary Fig. 3B and C). However, the protective effects of DsbA-L on diet-induced liver steatosis (Fig. 6D) and insulin resistance (Fig. 6E and F and Supplementary Fig. 3D and E) were markedly reduced in fDsbA-L/Ad−/− mice compared with fDsbA-L mice, suggesting that the protective effect of DsbA-L overexpression on diet-induced insulin resistance and liver steatosis is mainly mediated by adiponectin action. These results also suggest that HFD feeding could have an effect on insulin

FIG. 1. Expression of the myc-tagged DsbA-L transgene in mouse tissues. A: The fDsbA-L transgene construct. An aP2 promoter was used to drive the expression of myc-tagged mouse DsbA-L gene. B: Western blot analysis of tissue homogenates of the fDsbA-L transgenic mice using an anti-myc antibody. B, brain; F, fat; H, heart; L, liver; M, muscle; P, pancreas; S, spleen. The expression of the myc-tagged and endogenous DsbA-L in WAT (C), BAT (D), and macrophages (E) isolated from WT and fDsbA-L transgenic mice was analyzed by Western blot using an anti–DsbA-L antibody. Tubulin was used as a loading control.
resistance and liver dysfunction independent of its causative role in obesity.

The protective effect of fat-specific overexpression of DsbA-L on diet-induced obesity could be partially mediated by an adiponectin-independent mechanism. The above results suggest that enhanced adiponectin levels and multimerization play a major role in the insulin-sensitizing effect of DsbA-L in vivo. However, fat-specific overexpression of DsbA-L appears to have some additional beneficial effects in addition to promoting adiponectin multimerization and function. To further test this possibility, we compared the physiological and metabolic properties of the fDsbA-L/Ad"—/— and Ad"—/— littermates. There is no significant difference in food intake, body weight, and insulin sensitivity between fDsbA-L/Ad"—/— mice and Ad"—/— littermates under normal chow conditions (data not shown). However, the fDsbA-L/Ad"—/— mice displayed significantly less body weight and smaller fat cell size compared with Ad"—/— mice under HFD (Fig. 7A and B). In addition, the fDsbA-L/Ad"—/— mice were more active and displayed higher energy expenditure throughout the light and dark cycle compared with the Ad"—/— mice under HFD (Fig. 7C and D), suggesting that overexpression of DsbA-L had additional beneficial effects on diet-induced obesity through an
adiponectin-independent mechanism. However, diet-induced insulin resistance was similar in the fDsbA-L/AdΔ/Δ and AdΔ/Δ mice (Fig. 7E–H). Additionally, there was no significant difference in the glucose infusion rate and suppression of hepatic glucose production between fDsbA-L/AdΔ/Δ mice and the AdΔ/Δ mice fed with HFD (Supplementary Fig. 3F and G). Consistent with this, the fDsbA-L/AdΔ/Δ mice and AdΔ/Δ mice showed little difference in AMPK activity in both WAT and liver (data not shown). Taken together, these results suggest that adiponectin action plays a major role in mediating the protective effect of DsbA-L on diet-induced insulin resistance and liver steatosis.

DISCUSSION

Adiponectin is an anti–insulin resistant and anti-inflammatory adipokine that has great potential as a therapeutic target for various obesity-associated diseases such as type 2 diabetes, nonalcoholic steatohepatitis, and atherosclerosis (33). However, targeting adiponectin as a therapeutic intervention turns out to be difficult. Bacterially expressed full-length adiponectin, which lacks critical posttranslational modification, is essentially inactive (34), making large-scale production of this adipokine unfeasible. Efforts to increase adiponectin levels in vivo are also very challenging due to extremely high levels of endogenous adiponectin in vivo (35).

It has been shown that adiponectin oligomer distribution, rather than its absolute levels, correlates with a thiazolidinedione-mediated increase in insulin sensitivity (22). In addition, impairment in adiponectin multimerization has been shown to be associated with diabetes and hypo-adiponectinemia (4,6). These important findings suggest that promoting adiponectin multimerization rather than its total cellular levels could provide an effective approach for the treatment of obesity-related diseases. Several mechanisms, including hydroxylation (36), glycosylation (37), succination (38), and disulfide bond formation (7,39), have been found to regulate adiponectin multimerization. However, the key molecules regulating these modification processes remain largely unknown.

We have recently found that DsbA-L promotes adiponectin multimerization in 3T3-L1 adipocytes (26). In addition, overexpression of DsbA-L prevents ER stress–induced and autophagy-dependent downregulation of adiponectin in 3T3-L1 adipocytes (27). Although these results suggest that DsbA-L plays a key role in regulating adiponectin multimerization and stability, the in vivo function of this protein remains unknown. In the current study, we show that DsbA-L promotes adiponectin multimerization in vivo (Fig. 2). In addition, we have demonstrated that fat-specific overexpression of DsbA-L protects mice from HFD-induced adiponectin downregulation, insulin resistance, and hepatic steatosis (Figs. 2–5). These results not only demonstrate a critical role of DsbA-L in promoting adiponectin multimerization in vivo but also provide direct evidence to support the notion that enhancing adiponectin multimerization were counted per slide) using the NIH ImageJ program. D: Tissue composition of the HFD-fed fDsbA-L mice (n = 9) and WT littermates (n = 7) was analyzed by DEXA. The total activity (E) and oxygen consumption (F) of the HFD-fed fDsbA-L mice and WT littermates were measured during a 48-h period, including two complete light cycles and two complete dark cycles. The average of oxygen consumption was normalized to lean body mass. The data represent mean ± SEM. *P < 0.05 and **P < 0.01. (A high-quality digital representation of this figure is available in the online issue.)
could be an effective strategy for the treatment of obesity-associated metabolic diseases.

We previously found that incubation with DsbA-L alone was insufficient to promote adiponectin multimerization in vitro (26), suggesting that additional factors may be necessary for adiponectin multimerization in intact cells. Interestingly, adiponectin has been shown to be covalently bound to the ER chaperone ERp44 (39). Ero1-Lα (endoplasmic reticulum oxidoreductin 1–like protein α), another ER chaperone, promotes adiponectin release from ERp44 (7,39). It is possible that DsbA-L may promote the release of adiponectin from ERp44 by interacting with Ero1-Lα in cells, and thus facilitating adiponectin multimerization and secretion. Further studies will be needed to test this hypothesis.

The claim that adiponectin plays a role in regulating food intake has been controversial. Disruption of adiponectin gene expression did not significantly affect food intake and body weight (28,40,41). In addition, viral-mediated adiponectin expression ameliorated adiponectin deficiency–induced insulin resistance in liver or muscle but did not significantly affect the body weight of the mice (17,40). However, there is some data suggesting that the trimer form of adiponectin may function as a starvation hormone by regulating AMPK in the central nervous system to promote food intake (42). Although the origin of the low-molecular forms of adiponectin in the brain remains to be determined, the HMW form of this adipokine appears to be absent in the brain, probably due to the factor that formation of the HMW multimer prevents the crossing of the adipokine over the blood-brain barrier (42). Since overexpression of DsbA-L promotes the formation of the HMW form of adiponectin, it is conceivable that overexpression of DsbA-L has no major effect on food intake.

Our results suggest that enhanced energy expenditure may contribute to the antiobesity effect of DsbA-L. In agreement with this view, the activity of fDsbA-L mice was significantly increased compared with WT littermates. Additionally, the fDsbA-L mice displayed increased O2 consumption.
consumption compared with WT littermates (Fig. 3F). How fat-specific overexpression of DsbA-L leads to increased activity remains unknown, but acute peripheral administration of adiponectin has been shown to reduce body weight gain and visceral adiposity in obese mice, concurrently with enhanced rectal temperature and uncoupling protein 1 expression in BAT (43). In addition, adiponectin levels have been shown to be significantly correlated with thyroid hormones (44,45), which may contribute to increased activity. However, disruption of adiponectin expression only slightly impaired the effects of DsbA-L on body weight, fat mass, and energy expenditure on HFD (Fig. 6B and C and Supplementary Fig. 4B and C), suggesting that DsbA-L may have additional beneficial effects on energy homeostasis in addition to regulating adiponectin multimerization and

FIG. 4. Continued.

FIG. 5. Overexpression of DsbA-L in fat tissue reduced obesity-induced inflammation and hepatic steatosis. A: Macrophage infiltration into adipose tissue of HFD-fed fDsbA-L mice and control littermates as demonstrated by immunohistochemistry analysis with an anti-F4/80 antibody. Arrows: To point out infiltrated microphage. B: The mRNA levels of F4/80 and Mcp1 in gonadal WAT of the indicated mice were determined by quantitative real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as a control. n = 5–6 per group. C: Liver tissues were isolated from HFD-fed fDsbA-L and WT control mice, sectioned, and analyzed by Oil Red O staining. ND, normal diet. D: The triglyceride (TG) content in the liver of the fDsbA-L and WT control mice was determined using the triglyceride assay kit from Cayman Chemical Company and normalized to liver weight (wt). E: The expression levels of IL-6 and TNF-α in WAT of HFD-fed fDsbA-L and WT control mice were determined by Western blot and quantified by Scion Image Alpha 4.0.3.2 program. The expression levels were normalized to β-tubulin in each sample. n = 3. MCP1, monocyte chemoattractant protein-1. *P < 0.05; **P < 0.01. (A high-quality digital representation of this figure is available in the online issue.)
Consistent with this, HFD-fed fDsbA-L/Ad^−/− mice were leaner and more active compared with adiponectin-null mice, further suggesting an adiponectin-independent mechanism by which DsbA-L regulates energy homeostasis in vivo. It is possible that, in addition to adiponectin, DsbA-L may regulate the biosynthesis and secretion of other adipokines that exert an antiobesity effect. Alternatively, the overexpressed DsbA-L may enhance resistance to obesity through an autonomous action in adipocytes. Further investigations will be needed to test these possibilities.

Our results demonstrate that DsbA-L is an important regulator of adiponectin multimerization in vivo. Consistent with this, it has recently been found that whole-body knockout of DsbA-L (glutathione S-transferase [GST]−/−) slightly reduced serum adiponectin levels and caused glomerular nephropathy (46). However, very recently, the same group reported that knockout of DsbA-L/GST−/− had no effect on total adiponectin levels, adiponectin multimerization, insulin tolerance, and glucose tolerance compared with WT control mice under HFD (47). One possible
FIG. 7. DsbA-L could protect mice from HFD-induced obesity via an adiponectin-independent mechanism. Male fDsbA-L/Ad2/2 and Ad2/2 mice (7 weeks of age) were fed with a 45% HFD for 16 weeks. A: Body weight gains of the fDsbA-L/Ad2/2 and Ad2/2 mice. B: Representative H&E images showing fat cell size of epididymal fat from fDsbA-L/Ad2/2 and Ad2/2 male mice. The effects of DsbA-L overexpression on activity (C) and energy expenditure (D) were determined in a 48-h period including two dark and two light cycles. Oxygen consumption was normalized to lean body mass. E: The effects of DsbA-L overexpression on glucose tolerance in fDsbA-L/Ad2/2 and Ad2/2 mice. For GTT, 2 g/kg glucose was used. F: The AUC for the data shown in E was calculated using the trapezoidal rule. G: The effects of DsbA-L overexpression on insulin tolerance in fDsbA-L/Ad2/2 and Ad2/2 mice. For ITT, 0.075 units/kg insulin was used. The GTT and ITT data represent mean ± SEM. *P < 0.05 (ANOVA). H: The AUC for the data shown in G were calculated using the trapezoidal rule. The data represent mean ± SEM. *P < 0.05. (A high-quality color representation of this figure is available in the online issue.)
explanation for these controversies is that knockout of DsbA-L in vivo led to a compensatory increase in the expression of molecules that promote adiponectin expression and multimerization in adipose tissues. However, the finding that HFD feeding increased the levels of HMW adiponectin in both WT and DsbA-L knockout mice, which is contradictory to the findings of many others in the field (8,37,48–50), raises some concerns about the experimental conditions under which the experiments were performed. Thus, it remains to be determined whether knockout of DsbA-L under more physiologically relevant conditions affects adiponectin multimerization and function in vivo.

In summary, we have provided strong evidence for an in vivo role of DsbA-L in promoting adiponectin multimerization and function. Our study also demonstrates that enhanced adiponectin multimerization is sufficient to suppress obesity-induced insulin resistance and liver damage, suggesting that upregulation of DsbA-L could be an effective therapeutic approach for the treatment of obesity-induced insulin resistance and liver steatosis.

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M.L. and F.L. designed the experiment; researched data; wrote, reviewed, and edited the manuscript; and contributed to discussion. R.X. researched data and contributed to discussion. S.A.W., N.Z., L.B.S., K.A., L.Z., H.C., and G.X. researched data. C.A.W., S.N.A., N.M., R.A.D., and R.A. reviewed and edited the manuscript. P.E.S. and L.Q.D. reviewed and edited the manuscript and contributed to discussion. F.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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