Up-regulation of Adiponectin by Resveratrol

THE ESSENTIAL ROLES OF THE Akt/FOXO1 AND AMP-ACTIVATED PROTEIN KINASE SIGNALING PATHWAYS AND DsbA-L *

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The natural polyphenol resveratrol (RSV) displays a wide spectrum of health beneficial activities, yet the precise mechanisms remain to be fully elucidated. Here we show that RSV promotes the multimerization and cellular levels of adiponectin in 3T3-L1 adipocytes. The stimulatory effect of RSV was not affected by knocking out Sirt1, but was diminished by suppressing the expression levels of DsbA-L, a recently identified adiponectin-interactive protein that promotes adiponectin multimerization. Suppression of the Akt signaling pathway resulted in an increase in the expression levels of DsbA-L and adiponectin. On the other hand, knocking out FOXO1 or suppressing the activity or expression levels of the AMP-activated protein kinase (AMPK) down-regulated DsbA-L and adiponectin. The stimulatory effect of RSV on adiponectin and DsbA-L expression was completely diminished in FOXO1-suppressed mice (6, 7). The expression levels of DsbA-L are significantly reduced in obese human subjects and mice (6). On the other hand, the expression levels of DsbA-L are stimulated by the peroxisome proliferator-activated receptor γ agonist rosiglitazone (6). However, it is unknown whether DsbA-L expression could be stimulated by other insulin-sensitizing factors.

Resveratrol (RSV) is a polyphenol with antioxidant, anti-inflammatory, and anti-insulin resistance properties (8–11). Some studies suggest that the beneficial health effects of RSV are mainly mediated by the deacetylase Sirt1 (9–13). However, a number of recent studies demonstrate that RSV could exert its function via Sirt1-independent mechanisms (9, 14–16). There are some studies showing that RSV has a stimulatory effect on the expression levels (17, 18) or the secretion (19) of adiponectin. However, the precise underlying mechanisms remain largely unknown.

In the present report, we show that RSV stimulates adiponectin expression and multimerization in 3T3-L1 adipocytes via a Sirt1-independent mechanism. In addition, we demonstrate that the stimulatory effect of RSV is regulated by both the Akt/FOXO1 and the AMPK signaling pathways. Last, we show that DsbA-L plays a critical role in the promoting effect of RSV on adiponectin multimerization and cellular levels.

Adiponectin is an adipocyte-derived hormone that plays an important role in the regulation of insulin sensitivity and energy homeostasis. Adiponectin exists in cells and the plasma in three major forms: trimers, hexamers, and the high-molecular weight (HMW)3 forms, and the HMW form of adiponectin has been shown to be the most bioactive with respect to insulin action (1–3). Reduction of the HMW form, rather than the total levels of adiponectin, has been shown to be associated with various metabolic disease states (1, 3–5).

How adiponectin multimerization is regulated is not yet completely understood. We have recently identified an adiponectin interactive protein DsbA-L (disulfide bond-A oxidoreductase-like protein) that promotes adiponectin multimerization in 3T3-L1 adipocytes (6, 7). The expression levels of DsbA-L are significantly reduced in obese human subjects and mice (6). On the other hand, the expression levels of DsbA-L are stimulated by the peroxisome proliferator-activated receptor γ agonist rosiglitazone (6). However, it is unknown whether DsbA-L expression could be stimulated by other insulin-sensitizing factors.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—RSV, AICAR, and insulin were purchased from Sigma. Sirtinol, Akti VIII, and compound C were purchased from Calbiochem. Protein A-Sepharose beads were from Amersham Biosciences; anti-β-actin antibody was from BD Transduction Laboratories. Antisera to adiponectin were generated by our lab as described as previously (6). Antibodies to Sirt1, DsbA-L, and tubulin were from Upstate Biotechnology, PhosphoSolutions, and Sigma, respectively. All other antibodies were from Cell Signaling.

Cell Lines, Cell Culture, and Cell Differentiation—3T3-L1 CAR cells stably expressing a truncated receptor for coxsackievirus and adenovirus (CAR) was a gift of Dr. Jianhua Shao...
Western blot and Immunoprecipitation—The expression and phosphorylation levels of several proteins in cell lysates or immunoprecipitates were detected by Western blot with specific antibodies as described previously (21). Quantification of the Western blot.

Results

RSV Up-regulates DsbA-L and Adiponectin and Promotes Adiponectin Multimerization in 3T3-L1 Adipocytes and in Vivo—Treating 3T3-L1 adipocytes with RSV led to a time- and dose-dependent increase in the cellular levels of DsbA-L. Concurrently with the increase in DsbA-L expression levels, RSV treatment also led to a greater increase in the expression levels of adiponectin. Gel filtration experiments revealed that RSV treatment significantly increased the HMW form of adiponectin. Consistent with these findings, RSV treatment significantly enhanced the protein levels of DsbA-L and adiponectin in mouse adipose tissue.

DsbA-L Is Required for the Regulation of RSV on Adiponectin—To determine whether RSV-promoted multimerization and up-regulation of adiponectin is mediated by DsbA-L, we examined the effect of RSV in 3T3-L1 adipocytes in which the expression levels of DsbA-L are increased by overexpression or suppressed by RNAi. RSV treatment greatly stimulated the cellular levels of DsbA-L and adiponectin in the scramble 3T3-L1 adipocytes (Fig. 2A). The stimulatory effect of RSV on adiponectin expression, however, was markedly diminished in the DsbA-L-suppressed cells (Fig. 2A). On the other hand, overexpression of DsbA-L greatly increased adiponectin expression in 3T3-L1 adipocytes and RSV had no further stimulatory effect of RSV in the DsbA-L-overexp-
pressed cells (Fig. 2B). Gel filtration experiments revealed that RSV promoted adiponectin multimerization in the scramble 3T3-L1 adipocytes and the stimulatory effect of RSV was blocked in DsbA-L-suppressed cells (Fig. 2C). Taken together, these results suggest that DsbA-L plays a critical role in RSV-induced adiponectin multimerization and up-regulation.

**RSV Increases the mRNA Level of DsbA-L in 3T3-L1 Adipocytes**—To elucidate the mechanism by which RSV promotes DsbA-L and adiponectin up-regulation, we examined the effects of RSV on mRNA levels of DsbA-L and adiponectin in 3T3-L1 adipocytes by real time PCR. We found that RSV significantly increased the mRNA levels of DsbA-L, but had no significant effect on the mRNA levels of adiponectin (Fig. 2D). These results suggest that the promoting effect of RSV on adiponectin up-regulation is predominantly mediated by enhancing the expression levels of DsbA-L.

**The Stimulatory Effect of RSV on DsbA-L and Adiponectin Expression Is Independent of Sirt1**—To elucidate the signaling mechanism by which RSV promotes adiponectin multimerization and cellular levels, we asked whether the stimulatory effect of RSV on DsbA-L and adiponectin up-regulation is dependent on Sirt1, given that Sirt1 has been shown to mediate numerous functions of RSV (9, 10, 13). Inhibition of Sirt1 by sirtinol, as demonstrated by a marked increase in the acetylation levels of PGC-1α (Fig. 3A), had no significant effect on RSV-stimulated DsbA-L and adiponectin expression in 3T3-L1 adipocytes (Fig. 3A, the first and second panels). To further confirm the role of Sirt1 in regulation of RSV on DsbA-L and adiponectin expression, we examined the effect of RSV on the expression of DsbA-L and adiponectin in Sirt1-deficient adipocytes. RSV treatment enhanced the expression levels of DsbA-L and adiponectin in both wild-type and the
Sirt1-deficient adipocytes (Fig. 3B). Taken together, these results indicate that Sirt1 is dispensable for RSV-stimulated up-regulation of DsbA-L and adiponectin in adipocytes.

The Stimulatory Effect of RSV on DsbA-L and Adiponectin Expression Is Partially Mediated by Suppression of the PDK1/Akt Signaling Pathway—We have recently found that RSV treatment inhibits both mTORC1 and mTORC2 activity by promoting the interaction between mTOR and DEPTOR in C2C12 cells (20). Because activation of mTORC2 is critical for Akt phosphorylation at Ser473, which is critical for full activation of Akt, it is possible that RSV up-regulates DsbA-L and adiponectin by down-regulation of the Akt signaling pathway. To test this possibility, we examined whether RSV inhibits Akt phosphorylation in 3T3-L1 adipocytes. Treating 3T3-L1 adipocytes with RSV remarkably reduced insulin-stimulated Akt phosphorylation at Thr308 and Ser473, concurrently with a decrease in FOXO1 phosphorylation at Ser256, an Akt-phosphorylation site (Fig. 4A). To further determine the role of the Akt signaling pathway in the stimulatory effect of RSV, we examined DsbA-L expression levels in wild-type and PDK1-KO MEFs (we were unable to differentiate the PDK1-KO MEFs into adipocytes and thus only examined the effect of RSV on DsbA-L but not adiponectin expression). The expression levels of DsbA-L are greatly enhanced in the PDK1-KO MEFs compared with wild-type cells (Fig. 4B), suggesting that the PDK1/Akt signaling pathway plays a negative role in regulation of DsbA-L expression. Interestingly, RSV treatment led to a further increase in the expression levels of DsbA-L (Fig. 4B), suggesting that, in addition to inhibition of

FIGURE 3. Resveratrol up-regulates DsbA-L and adiponectin expression is independent of Sirt1. A, 3T3-L1 adipocytes were pre-treated with or without 10 μM sirtinol for 60 min, followed with or without 50 μM RSV for 24 h. The acetylation and protein levels of immunoprecipitated PGC-1α were determined by Western blot with specific antibodies. B, the differentiated Sirt1-KO/WT adipocytes were treated with RSV at the indicated concentrations for 24 h. For A and B, the expression levels of adiponectin and DsbA-L in cell lysates were determined by Western blot using specific antibodies.

FIGURE 4. The stimulatory effects of RSV on DsbA-L and adiponectin expression are partially through suppression of the PI3K pathway. A, serum-starved 3T3-L1 adipocytes were pre-treated with or without 50 μM RSV for 20 min and then with or without 10 nM insulin for 10 min. The phosphorylation of Akt at Thr308, Ser473, and Foxo1 at Ser256 and the protein levels of these kinases in cell lysates were determined by Western blot using antibodies as indicated. B, PDK1-KO/WT MEF cells were treated with or without 50 μM RSV for 24 h. C, 3T3-L1 adipocytes were pre-treated with or without 10 μM Akt inhibitor VIII for 60 min, followed with or without 50 μM RSV for 24 h. D, Foxo1-suppressed or scramble cells were treated with or without 50 μM RSV for 24 h. For B–D, the expression levels of DsbA-L, adiponectin, PDK1, Akt, Foxo1, and phosphorylation levels of Akt (Thr308) and Foxo1 (Ser256) in cell lysates were determined by Western blot using antibodies as indicated. The data are representative of three independent experiments with similar results and quantified by the NIH Scion Image program. Data are presented as mean ± S.E. Differences between groups were tested for statistical significance using analysis of variance. *, p < 0.05; **, p < 0.01.
the PDK1/Akt signaling pathway, RSV may up-regulate DsbA-L by acting on a novel target.

To further determine the role of the Akt signaling pathway in regulation of DsbA-L and adiponectin expression, we treated 3T3-L1 adipocytes with an Akt-specific inhibitor, AKTi VIII. As expected, AKTi VIII treatment inhibited the phosphorylation of Akt and FOXO1 concurrently with an increase in the expression levels of DsbA-L and adiponectin (Fig. 4C). The expression levels of DsbA-L and adiponectin were greatly reduced in FOXO1-suppressed 3T3-L1 adipocytes compared with the scramble cells (Fig. 4D). Suppressing the cellular levels of FOXO1 greatly reduced basal and RSV-induced expression levels of DsbA-L and adiponectin (Fig. 4D). Consistent with the view that there is an Akt/FOXO1-independent mechanism by which RSV enhances DsbA-L and adiponectin cellular levels, RSV was still able to promote DsbA-L and adiponectin expression in the FOXO1-suppressed cells, although the stimulatory effect was much smaller compared with that observed in the scramble cells (Fig. 4D).

The AMPK Signaling Pathway Is Involved in RSV-stimulated Up-regulation of DsbA-L and Adiponectin—To elucidate the Akt-independent mechanism underlying RSV-induced DsbA-L and adiponectin up-regulation, we asked whether AMPK, which has been shown to be activated by RSV (14, 29, 30), plays a role in RSV-stimulated up-regulation of DsbA-L and adiponectin. Axon stimulation of AMPK by AICAR markedly enhanced the protein levels of DsbA-L and adiponectin in 3T3-L1 adipocytes (Fig. 5C). However, AICAR co-treatment had no further effect on RSV-stimulated DsbA-L and adiponectin up-regulation (Fig. 5C). Consistent with this data, treating 3T3-L1 adipocytes with Compound C, a specific inhibitor of AMPK (31), had little effect on the basal expression of DsbA-L and adiponectin (Fig. 5A). However, treating 3T3-L1 adipocytes with Compound C significantly suppressed RSV-stimulated expression of DsbA-L and adiponectin (Fig. 5A). Furthermore, RSV stimulated DsbA-L expression in scramble C2C12 cells and the stimulatory effect of RSV was significantly reduced in C2C12 cells in which the expression of AMPK is suppressed by RNAi (Fig. 5B). To further confirm the necessity of the AMPK and FOXO1 signaling pathways in RSV-stimulated DsbA-L and adiponectin up-regulation, we asked whether AMPK, which has been shown to be activated by RSV (14, 29, 30), plays a role in RSV-stimulated up-regulation of DsbA-L and adiponectin. Axon stimulation of AMPK by AICAR markedly enhanced the protein levels of DsbA-L and adiponectin in 3T3-L1 adipocytes (Fig. 5C). However, AICAR co-treatment had no further effect on RSV-stimulated DsbA-L and adiponectin up-regulation (Fig. 5C). Consistent with this data, treating 3T3-L1 adipocytes with Compound C, a specific inhibitor of AMPK (31), had little effect on the basal expression of DsbA-L and adiponectin (Fig. 5A). However, treating 3T3-L1 adipocytes with Compound C significantly suppressed RSV-stimulated expression of DsbA-L and adiponectin (Fig. 5A). Furthermore, RSV stimulated DsbA-L expression in scramble C2C12 cells and the stimulatory effect of RSV was significantly reduced in C2C12 cells in which the expression of AMPK is suppressed by RNAi (Fig. 5B).
tin up-regulation, we treated scramble and FOXO1-RNAi 3T3-L1 CAR adipocytes with Compound C. Compound C treatment partially suppressed the stimulatory effect of RSV on the expression levels of DsbA-L and adiponectin in the scramble 3T3-L1 adipocytes (Fig. 5D). The promoting effect of RSV was completely diminished in the FOXO1-RNAi cells treated with Compound C (Fig. 5D), demonstrating that activation of FOXO1 and the AMPK signaling pathway are the major mechanisms by which RSV promotes DsbA-L and adiponectin expression.

**DISCUSSION**

RSV has various health beneficial roles, yet the molecular mechanisms by which RSV exerts its biological function remain to be fully elucidated. Some studies demonstrate that RSV exerts its roles by activation of the NAD$^+$-dependent deacetylase Sirt1 (8, 9, 25, 32). However, a number of recent studies have shown that RSV regulates many of the biological events such cell growth, glucose homeostasis, and protection of the cardiovascular system via a Sirt1-independent mechanism (9, 14, 33–35). In the present study, we show that RSV promotes adiponectin multimerization and up-regulation via a Sirt1-independent mechanism. Because adiponectin oligomer distribution is crucial for its biological functions (3, 6) and adiponectin mutants with impaired multimerization are defective in both secretion and are associated with insulin resistance and hypoadiponectinemia (1, 5), our results thus provide a new mechanism by which RSV exerts its beneficial roles in anti-inflammation, anti-insulin resistance, and cardioprotective functions.

We have shown that RSV stimulates the expression levels of DsbA-L, a recently identified protein that facilitates adiponectin multimerization and stability in cells (6, 25). In addition, we found that the stimulatory effects of RSV on adiponectin cellular levels and multimerization are diminished in DsbA-L-suppressed 3T3-L1 adipocytes (Fig. 2, A and C). Taken together with the finding that RSV had no significant effect on the mRNA levels of adiponectin (Fig. 2D), it is conceivable that the effects of RSV on adiponectin multimerization and up-regulation are mainly mediated by up-regulation of DsbA-L. Consistent with this view, overexpression of DsbA-L has been shown to enhance the cellular levels of adiponectin in 3T3-L1 adipocytes (6) and prevent endoplasmic reticulum stress-induced adiponectin down-regulation (25).

Several recent studies suggest that the PI3K/PDK1/Akt signaling pathway is involved in the action of RSV to regulate metabolism (36, 37). However, it is unknown whether this pathway plays a role in RSV-stimulated adiponectin and DsbA-L expression. We have found that inhibition of this signaling pathway increased the cellular levels of DsbA-L and adiponectin in 3T3-L1 adipocytes. In addition, we show that up-regulation of DsbA-L and adiponectin is partially mediated by activation of FOXO1, a transcription factor that has been shown to play a role in regulating adiponectin gene expression (26–28). However, we found that RSV treatment had no significant effect on the mRNA levels of adiponectin (Fig. 2D). This result is somewhat inconsistent with the finding of Qiao and Shao (38) who found that activation of FOXO1 promotes adiponectin transcription. The exact reason for this discrepancy remains unknown but FOXO1 has been found to suppress peroxisome proliferator-activated receptor γ gene expression (39). Because peroxisome proliferator-activated receptor γ positively regulates adiponectin gene expression and secretion, these findings suggest that the effects of FOXO1 on adiponectin biosynthesis may depend on cell content and upstream signaling events. Alternatively, because treating the cells with RSV significantly increased the mRNA levels of DsbA-L (Fig. 2D), it is possible that the major role of RSV on adiponectin up-regulation is mediated by DsbA-L. Consistent with this, increased cellular levels of DsbA-L have been shown to increase the multimerization and stability of adiponectin (6, 25).

In addition to the Akt/FOXO1 signaling pathway, our work demonstrates that the AMPK signaling pathway also plays a key role in the stimulatory effect of RSV on DsbA-L and adiponectin expression. The stimulatory effect of RSV on adiponectin expression is completely suppressed in AMPK-inhibited and FOXO1-suppressed cells (Fig. 5D), implicating that the Akt/FOXO1 and the AMPK signaling pathways are the two major pathways mediating the stimulatory effect of RSV on DsbA-L and adiponectin expression. However, how RSV activates AMPK in 3T3-L1 adipocytes remains unknown, although some recent studies showed that RSV increase AMP levels by inhibition of the mitochondrial F1 ATPase in neuronal cells (35, 40). Further studies will be needed to determine whether a similar mechanism is operative in adipocytes.

In summary, we have shown that RSV plays a positive role in regulating adiponectin expression and multimerization in adipocytes via a Sirt1-independent mechanism. In addition, we have demonstrated that the stimulatory effects of RSV are mediated mainly by suppressing the PDK1/Akt signaling pathway, which results in FOXO1 activation, and by activation of the AMPK signaling pathway. Our studies show that RSV treatment had little effect on the mRNA levels of adiponectin, but significantly enhanced the mRNA and protein levels of DsbA-L, suggesting that the promoting effects of RSV on adiponectin multimerization and expression are mainly mediated by up-regulating DsbA-L. Because enhancing adiponectin levels increases resistance to inflammation, insulin resistance, and cardiovascular disorders, the finding that RSV promotes adiponectin expression levels thus provide a novel mechanism by which RSV exerts its health beneficial functions.

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Up-regulation of Adiponectin by Resveratrol

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