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Adiponectin Is Required for PPARγ-Mediated Improvement of Endothelial Function in Diabetic Mice

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SUMMARY

Rosiglitazone is a PPARγ agonist commonly used to treat diabetes. In addition to improving insulin sensitivity, rosiglitazone restores normal vascular function by a mechanism that remains poorly understood. Here we show that adiponectin is required to mediate the PPARγ effect on vascular endothelium of diabetic mice. In db/db and diet-induced obese mice, PPARγ activation by rosiglitazone restores endothelium-dependent relaxation of aortae, whereas diabetic mice lacking adiponectin or treated with an anti-adiponectin antibody do not respond. Rosiglitazone stimulates adiponectin release from fat explants, and subcutaneous fat transplantation from rosiglitazone-treated mice recapitulates vasodilatation in untreated db/db recipients. Mechanistically, adiponectin activates AMPK/eNOS and cAMP/PKA signaling pathways in aortae, which increase NO bioavailability and reduce oxidative stress. Taken together, these results demonstrate that adipocyte-derived adiponectin is required for PPARγ-mediated improvement of endothelial function in diabetes. Thus, the adipose tissue represents a promising target for treating diabetic vasculopathy.

INTRODUCTION

Obesity and diabetes are common risk factors for the initiation of vascular dysfunction. Adipose tissue is now recognized as an important metabolic and endocrine organ in the regulation of glucose metabolism. Dysregulation of adipose tissue participates in the development of insulin resistance and vascular complications of diabetes (Hajer et al., 2008).

The gene expression pattern of adiponectin in subcutaneous and visceral adipose tissue and the levels of circulating adipokines predict insulin resistance and diabetic risk (Samaras et al., 2010). Although obesity is a common contributor to insulin resistance, the molecular link between increased adiposity and impaired vascular function in humans is not fully elucidated. Adipose tissue also contributes to the regulation of vascular tone (Fésüs et al., 2007; Gálvez-Prieto et al., 2008; Verlohren et al., 2004). Chronic inflammation of adipose tissue leads to vascular dysfunction due to a diminished production of vasoprotective cytokines and increased release of inflammatory cytokines by adipocytes. However, the role, if any, of adipose tissue in vascular benefit of antidiabetic drugs is largely unclear.

Adiponectin is an adipose-secreted protein that exerts both antiatherogenic and insulin-sensitizing effects, and a reduced production of adiponectin is closely coupled to insulin resistance (Kadowaki et al., 2006; Whitehead et al., 2006; Zhu et al., 2008). The plasma concentration of adiponectin in obese subjects is lower than that in nonobese subjects and inversely correlates with body mass index (Arita et al., 1999). Moreover, hypoadiponectinemia is associated with reduced endothelium-dependent dilatation in both diabetic and nondiabetic human subjects (Ouchi et al., 2003; Shimabukuro et al., 2003; Tan et al., 2004; Torigoe et al., 2007). The nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) is a major regulator of adipocyte function and controls the secretion of adipokines, in particular promoting the production of adiponectin (Anghel et al., 2007; Crossno et al., 2006; Maeda et al., 2001) while limiting the generation of proinflammatory TNF-α, IL-6, and IL-1β (Jiang et al., 1998). The insulin-sensitizing drugs thiazolidinediones (TZDs), including rosiglitazone and pioglitazone, are high-affinity ligands that act on PPARγ in liver, skeletal muscle, and adipose tissue. TZDs also increase plasma adiponectin levels in insulin-resistant humans (Yang et al., 2002; Zhu et al., 2008). PPARγ ligands improve endothelial function through multiple mechanisms, including stimulation of eNOS (Calnek et al., 2003; Cho et al., 2004; Yasuda et al., 2009), inhibition of inflammatory target

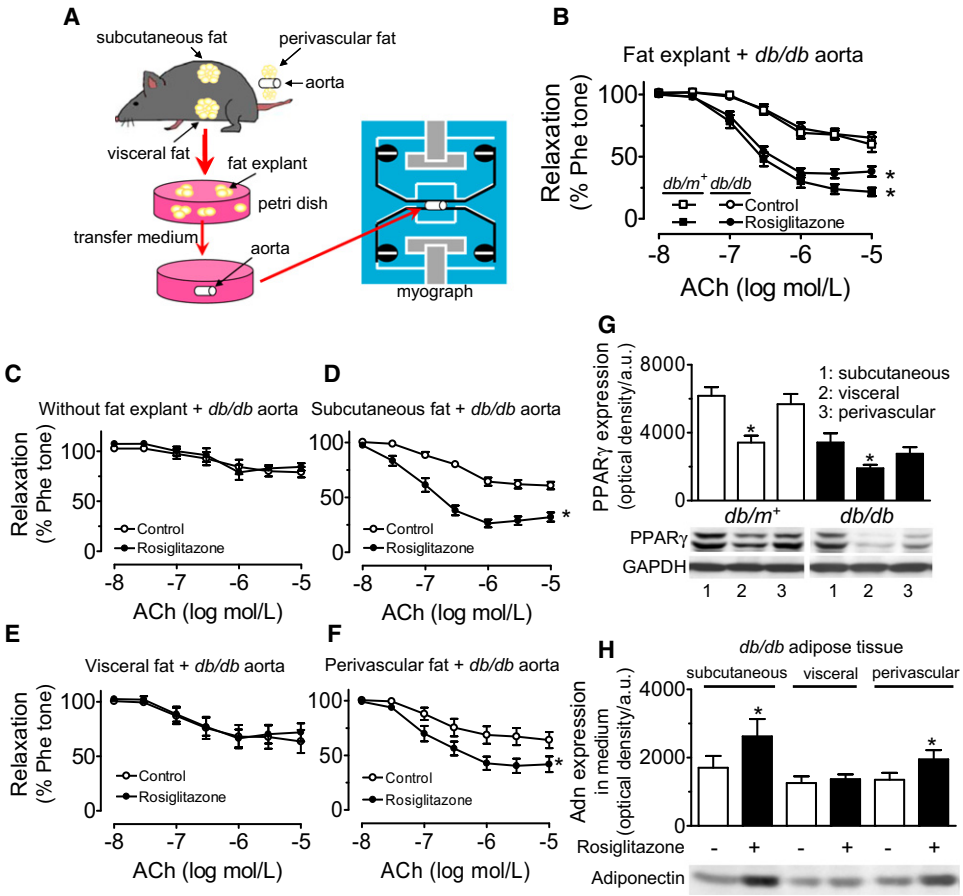

Figure 1. Adipose Tissue Is Required for PPARγ Activation-Induced Amelioration of the Impaired Endothelium-Dependent Relaxations in *db/db* Mouse Aortae

(A) Procedure of fat explant experiments.

(B) Acetylcholine-induced endothelium-dependent relaxations in *db/db* aortae after incubation in culture medium from rosiglitazone-treated fat explants (pool of subcutaneous, visceral, and perivascular fats) from *db/db* and *db/m*$^+$ mice.

(C) Effect of 12 hr exposure of *db/db* mouse aortae to 1 μmol/l rosiglitazone alone. Results are means ± SEM of 6–8 experiments from different mice.

(D) Effect of 12 hr incubation of fat explant from subcutaneous adipose tissue from *db/db* mouse with 1 μmol/l rosiglitazone. Results are means ± SEM of 6–8 experiments from different mice.

(E) Effect of 12 hr incubation of fat explant from visceral adipose tissue from *db/db* mouse with 1 μmol/l rosiglitazone. Results are means ± SEM of 6–8 experiments from different mice.

(F) Effect of 12 hr incubation of fat explant from perivascular adipose tissue from *db/db* mouse with 1 μmol/l rosiglitazone. Results are means ± SEM of 6–8 experiments from different mice.

(G) The protein expression of PPARγ in subcutaneous, visceral, and perivascular adipose tissues from *db/m*$^+$ and *db/db* mice. Results are means ± SEM of four experiments.

(H) The levels of adiponectin present in culture medium after incubation of subcutaneous, visceral, and perivascular fat explants with rosiglitazone. Results are means ± SEM of four experiments. *p < 0.05 versus control within each group.

genes (Kanda et al., 2009; Lee et al., 2009; Orasanu et al., 2008), and downregulation of NAD(P)H oxidases (Ceolotto et al., 2007).

Although TZDs are widely used to restore insulin sensitivity in patients with type 2 diabetes (Yki-Järvinen, 2004), the molecular mechanisms that confer their vascular protection are poorly understood. The present experiments were designed to test the hypothesis that adipocyte-derived adiponectin plays an indispensable role in the amelioration of endothelial dysfunction in diabetes following chronic treatment with PPARγ agonists. The present results demonstrate that subcutaneous adipose tissue is an important target for PPARγ agonists to improve diabetic endothelial function. Adipocyte-derived adiponectin preserves the bioavailability of nitric oxide (NO) by two independent cellular mechanisms.

RESULTS

Adipose Tissue Is Required for PPARγ Activation-Induced Restoration of Endothelial Function in *db/db* Mouse Aortae

In order to reveal the role of adipose tissue in PPARγ-induced endothelial protection in vivo, an ex vivo fat explant organ culture method is used to examine the effect of adipokines from different fat depots on vascular function (Figure 1A). Rosiglitazone

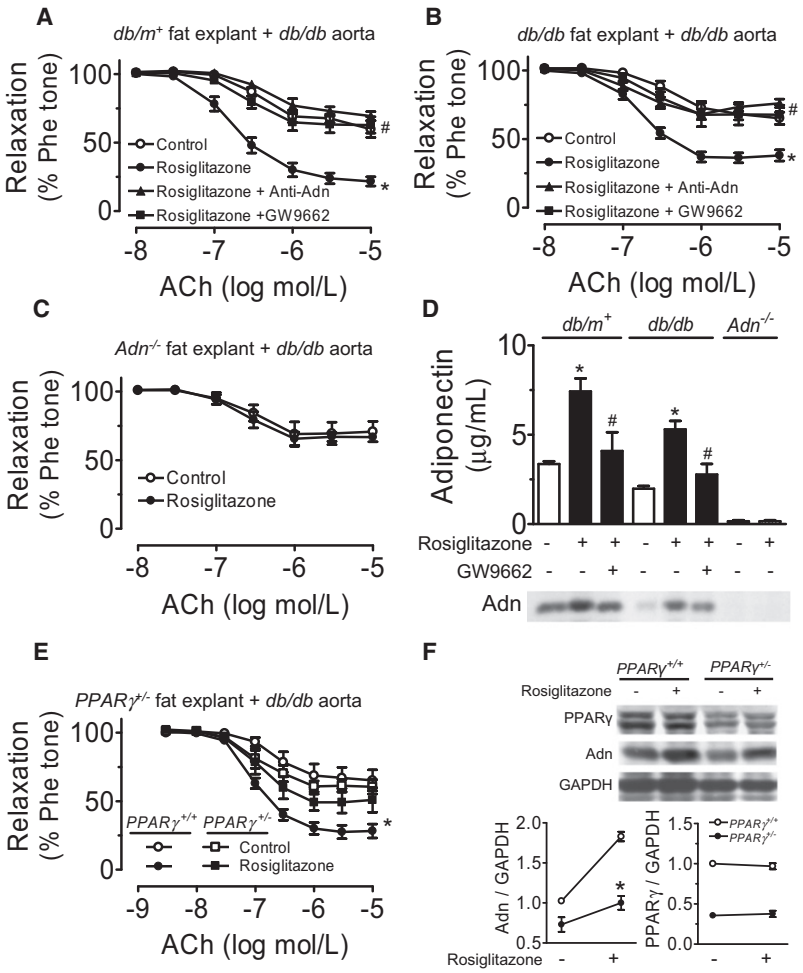

Figure 2. Role of Adiponectin in PPARγ Agonist-Induced Vascular Benefit in *db/db* Mice

(A and B) Anti-adiponectin antibody (anti-Adn, 5 μg/ml) and GW9662 (5 μmol/l, PPARγ antagonist) abolished the effect of PPARγ-activated subcutaneous fat explants by rosiglitazone (*db/m*$^+$, A; *db/db*, B) to improve the EDR in aortae from *db/db* mice.

(C) EDR of aortae from *db/db* mice did not improve after incubation in medium of fat explants from *adiponectin*$^{-/-}$ (*Adn*$^{-/-}$) mice treated with rosiglitazone.

(D) The levels of adiponectin present in culture medium from fat explants under identical procedures as described in (A)–(C).

(E) Effect of rosiglitazone-treated subcutaneous fat explants from *PPARγ*$^{+/-}$ mice on EDR in aortae from *db/db* mice.

(F) The expression of PPARγ and adiponectin release from fat explants of *PPARγ*$^{+/-}$ mice. Results are means ± SEM of 4–6 experiments. *p < 0.05 versus control, #p < 0.05 versus rosiglitazone.

(1 μmol/l, 12 hr)-activated adipose tissue (pool of subcutaneous, visceral, and perivascular fats) from either *db/m*$^+$ or *db/db* mice significantly improved endothelium-dependent relaxations (EDRs) in *db/db* mouse aortae (Figure 1B), while 12 hr exposure of isolated *db/db* mouse aortae to rosiglitazone alone did not improve EDRs (Figure 1C). The EDRs were increased markedly only by medium from rosiglitazone-activated subcutaneous adipose tissue (Figure 1D), while medium from visceral adipose tissue had no effect (Figure 1E), and that from perivascular adipose tissue caused a moderate potentiation of EDRs (Figure 1F). Endothelium-independent relaxations to sodium nitroprusside (SNP) were similar in aortic rings from all the groups (Figure S1A).

Adipose tissue from *db/db* mice expressed significantly less PPARγ. The expression level of PPARγ corresponded to the effect on EDRs from different fat depots of *db/db* and *db/m*$^+$ mice. Subcutaneous fat expressed the highest level of PPARγ, followed by perivascular fat, while visceral fat contained the least amount of PPARγ (Figure 1G). The adiponectin release in response to rosiglitazone in subcutaneous fat was significantly higher than in perivascular or visceral fat from *db/db* mice (Figure 1H). The following experiments were performed using only subcutaneous fat from *db/db* mice in organ culture.

Adiponectin and Vascular Effects of Rosiglitazone

We next tested the effects of PPARγ antagonist GW9662 and anti-adiponectin neutralizing antibody to establish an essential role of adipose tissue-derived adiponectin in mediating PPARγ-dependent improvement of EDRs in *db/db* mice. Both anti-adiponectin antibody (5 μg/ml) and GW9662 (5 μmol/l) prevented the effect of PPARγ-activated fat explant from *db/db* (Figure 2A) and *db/m*$^+$ mice (Figure 2B). The role of adipocyte-derived adiponectin was further confirmed as EDR in *db/db* mice was not restored by rosiglitazone-treated fat explant from *Adn*$^{-/-}$ mice (Figure 2C). Rosiglitazone elevated the amounts of adiponectin released by fat explants from *db/db* and *db/m*$^+$ mice but not from *Adn*$^{-/-}$ mice, and this increase was inhibited by GW9662 (Figure 2D). Finally, we verified the role of PPARγ using *PPARγ*$^{+/-}$ mice. Rosiglitazone-stimulated adiponectin release was much less in fat explants from *PPARγ*$^{+/-}$ mice, which might account for small improvement of EDRs in *db/db* mouse aortae, correlating with less PPARγ expression (Figures 2E and 2F).

Fat Transplantation In Vivo Improves Endothelial Function in Diabetic Mice

To further confirm the importance of subcutaneous fat in endothelial protection in response to PPARγ activation, we performed fat transplantation experiments in diabetic mice. This was done using subcutaneous flank fat of donor *db/db* mice, removing a similar amount of subcutaneous fat of recipient *db/db* mice, and placing the fat grafts into the same dorsal subcutaneous area from the donor to the recipient (Figure 3A). Some donors or recipients were treated with rosiglitazone (10 mg/kg/day for 4 weeks) prior to fat transplantation. EDRs were improved in control recipient mice receiving fat graft from rosiglitazone-treated mice (C+RF) (Figure 3B). Rosiglitazone-treated recipient mice receiving fat graft from either rosiglitazone-treated donors

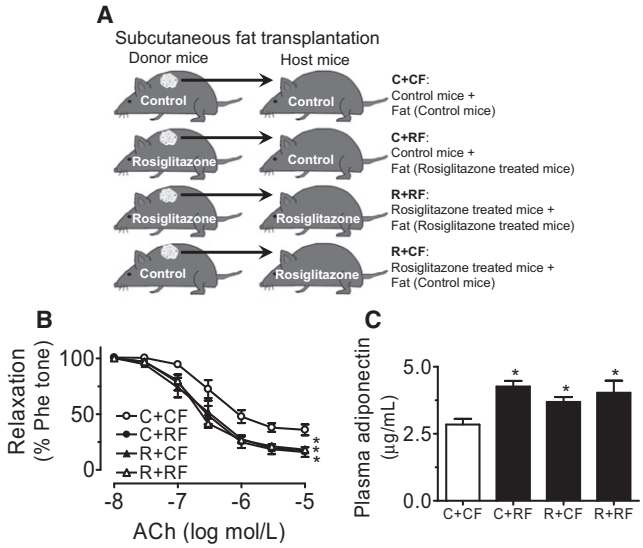

Figure 3. Fat Transplantation from Rosiglitazone-Treated *db/db* Mice Improves Endothelial Function in Control *db/db* Mice

(A) Schematic of fat transplantation procedure.

(B) Improved EDR observed in aortae from rosiglitazone-treated *db/db* mice receiving fat grafts from either control (R+CF) or rosiglitazone-treated *db/db* mice (R+RF) and also from control *db/db* mice receiving fat grafts from rosiglitazone-treated *db/db* mice (C+RF), compared with the impaired EDR from control *db/db* mice (C+CF). Results are means ± SEM of six mice. *p < 0.05 versus C+CF.

(C) Plasma adiponectin level in all the groups of *db/db* mice. Results are means ± SEM of 3–4 mice. *p < 0.05 versus C+CF.

(R+RF) or from control donors (R+CF) had EDRs similar to C+RF littermates, which were also improved compared with those from control recipients receiving fat grafts from control donor mice (C+CF) (Figure 3B). Plasma adiponectin concentration also increased significantly in C+RF, R+CF, and R+RF groups compared to those from C+CF group (Figure 3C).

In Vivo Rosiglitazone Treatment Improves Endothelial Function via Adiponectin-Dependent Mechanisms

EDRs in aortae were reduced in *db/db* compared to *db/m*$^{+}$ mice (Figures 4A and 4B). Administration of *db/db* mice with rosiglitazone (10 mg/kg body weight/day) for 4 weeks potentiated EDRs in their aortae, but not in those from *db/Adn*$^{-/-}$ *DKO* mice (Figures 4A and 4B), while the glucose tolerance test showed similar improvement in both *db/db* and *db/Adn*$^{-/-}$ *DKO* mice after rosiglitazone treatment (Figures 4C and 4D). Rosiglitazone treatment in *db/db* mice also increased the plasma adiponectin level (2.86 ± 0.22 μg/ml in vehicle-treated mice versus 8.08 ± 1.45 μg/ml in rosiglitazone-treated mice, $p < 0.05$). Aortae from *db/db* mice exhibited fewer phosphorylations of AMPKα at Thr172 and eNOS at Ser1177 compared with those from *db/m*$^{+}$ mice, which were restored by in vivo rosiglitazone treatment (Figures 4E–4G).

Aortae from *db/db* mice showed less expression of AdipoR2 but not AdipoR1 compared to those from *db/m*$^{+}$ mice (Figures 4E, 4H, and 4I). Rosiglitazone treatment increased AdipoR2 expression in aortae from *db/db* mice without affecting AdipoR1 expression (Figures 4E, 4H, and 4I).

Effect of PPARγ and Adiponectin in Diet-Induced Obese Mice

To verify the effect of rosiglitazone in another mouse model of diabetes, experiments were performed in high-fat-diet-induced obese (DIO) mice. Aortae from DIO mice exhibited reduced EDR compared with age-matched C57BL/6J littermates, while endothelium-independent relaxations to SNP were unaltered (Figure S1B). After 12 hr incubation in medium collected from fat explants of DIO mice treated with rosiglitazone, EDRs were improved (Figure 5A) and were inhibited by GW9662. Improved EDRs correlated to the adiponectin concentrations in the medium (Figure 5B). Similar results were obtained with troglitazone or ciglitazone treatment (Figures S2B–S2D). Rosiglitazone treatment in vivo of DIO mice (10 mg/kg/day, 2 weeks) normalized the EDRs in their aortae and increased plasma adiponectin level (Figures 5C and 5E). In addition, the improved EDRs after in vivo rosiglitazone treatment were inhibited by overnight incubation with compound C or anti-adiponectin antibody, but not by GW9662 (Figure 5D). The reduced phosphorylation of AMPKα and eNOS in aortae from DIO mice increased after rosiglitazone treatment (Figures 5F and 5G).

Adiponectin Increases NO Bioavailability through AMPK and PKA

Adiponectin (5 μg/ml) augmented EDR in *db/db* mouse aortae (Figure 6A). Such effect was abolished by anti-adiponectin antibody (Figure 6A) and inhibited by compound C (Figure 6B), but unaffected by GW9662 (Figure 6A). A similar effect was also observed using aortae from DIO mice (Figure S3). Treatment with PKA inhibitors, H89 (1 μmol/l) or Rp-cAMP (10 μmol/l), and adenylyl cyclase inhibitor SQ22536 (10 μmol/l) attenuated the effect of adiponectin (Figure 6C). Combined treatment with compound C and H89 did not cause further inhibition (Figure 6B). Adiponectin increased the phosphorylation of AMPKα at Thr172 and eNOS at Ser1177. Compound C but not H89 inhibited adiponectin-stimulated AMPKα phosphorylation (Figure 6D). Compound C and SQ22536 also attenuated adiponectin-stimulated eNOS phosphorylation (Figure 6E).

The aortae of *db/db* mice contained higher levels of ROS compared with those of *db/m*$^{+}$ mice, as revealed by DHE fluorescence staining. Treatment with adiponectin reduced ROS, and this effect was abolished by H89 or SQ22536, but not by compound C (Figures 7A and 7B). Forskolin, a cyclic AMP-elevating agent (100 nmol/l), produced an effect similar to adiponectin in reducing ROS (Figures 7A and 7B). Both adiponectin and forskolin raised the tissue content of cyclic AMP in *db/m*$^{+}$ and *db/db* mouse aortae. Only SQ22536, but not the other inhibitors, prevented the adiponectin-induced increase in cyclic AMP level (Figure 7C).

The NO production in response to A23187 (1 μmol/l) was blunted in primary mouse aortic endothelial cells incubated in high glucose (30 mmol/l, HG) when compared with mannitol control (NG) (Figures 7D and 7E). Incubation of 5 μg/ml adiponectin restored the reduced NO production under high-glucose condition (Figures 7D and 7E).

DISCUSSION

The present study defines an obligatory role of adipose tissue, particularly subcutaneous fat depots, in an improvement of

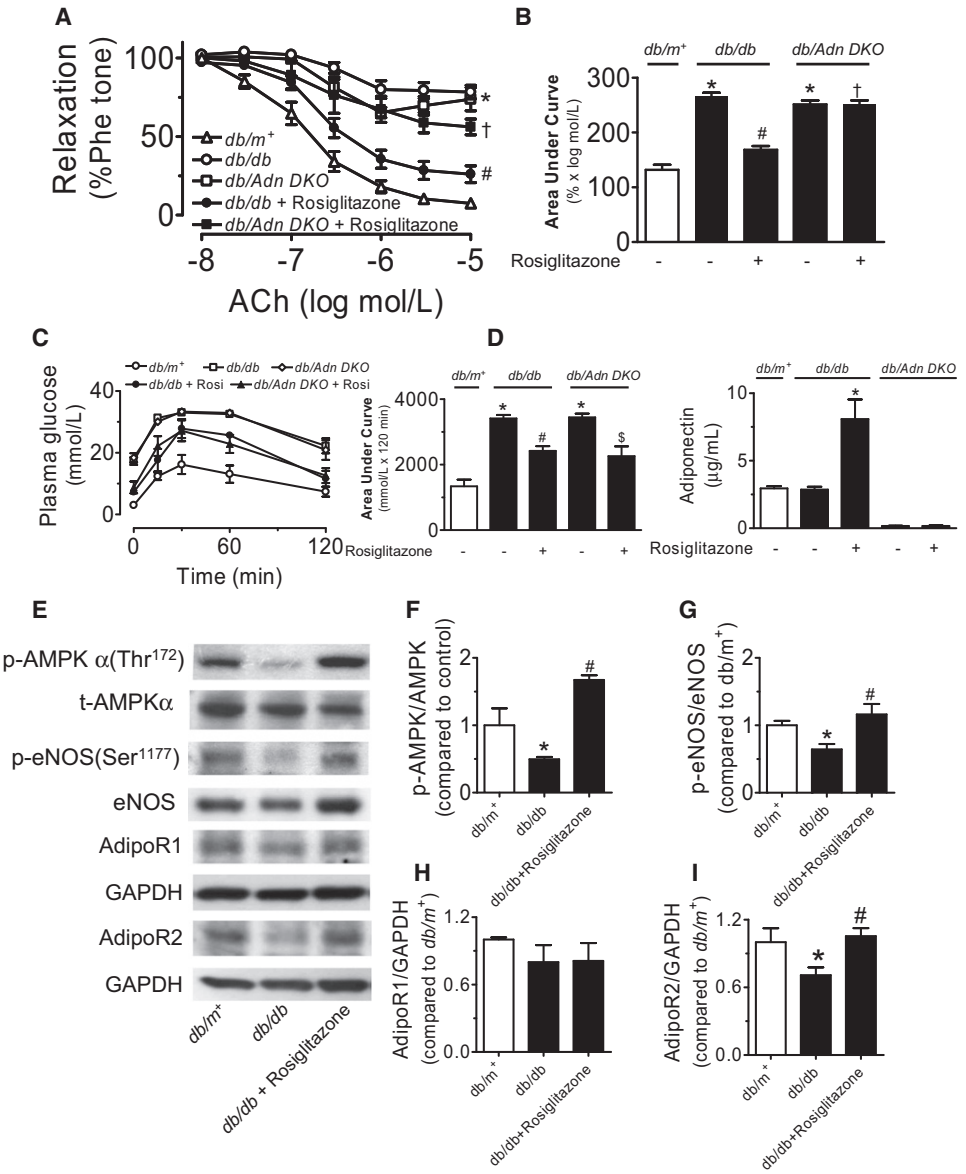

Figure 4. In Vivo Rosiglitazone Treatment Improves Endothelial Function in Diabetic Mice through Adiponectin-Dependent Mechanism

(A and B) Chronic treatment with rosiglitazone improved EDR in aortae from *db/db* mice. Potentiation of EDR was abolished in aortae from *db/Adn DKO* mice.

(C and D) Oral glucose tolerance test showed that rosiglitazone treatment improved glucose tolerance in both *db/db* and *db/Adn DKO* mice.

(E–G) Western blotting showed the AMPKα and eNOS phosphorylation with total AMPKα and eNOS levels in aortae from *db/db* mice after rosiglitazone treatment.

(H and I) Western blotting showed the expressions of AdipoR2 and AdipoR1 in aortae from the three groups of mice. Results are means ± SEM of six mice. *p < 0.05 versus *db/m*$^+$, #p < 0.05 versus *db/db*, †p < 0.05 versus *db/db* + Rosiglitazone, and $p < 0.05 versus *db/Adn DKO*.

endothelial function in diabetic mice following PPARγ activation. It demonstrates that adipocyte-derived adiponectin is the primary mediator that improves endothelial function and does so by both AMPK- and PKA-dependent mechanisms. The present findings suggest that adipose tissue can be an important therapeutic target in the protection of vascular dysfunction in diabetes by producing and releasing anti-inflammatory vasoactive hormones, among which adiponectin plays an indispensable role in protecting vascular function.

The present study employs multiple approaches, aided by the use of relevant genetically modified mice, to demonstrate a crucial role of adipocyte-derived adiponectin in PPARγ agonist-induced endothelial cell protection in *db/db* and DIO mice. Differential expression levels of PPARγ in the three fat depots were observed, with PPARγ expression being most abundant in subcutaneous, intermediate in perivascular, and least in visceral adipose tissues. The PPARγ level positively correlated with the amount of adiponectin released upon PPARγ activation and the extent of improvement of EDR in aortae from *db/db* mice. The present results indicate that PPARγ agonists do not act on the endothelium directly, since exposure to rosiglitazone did not augment the EDR without the presence of adipose

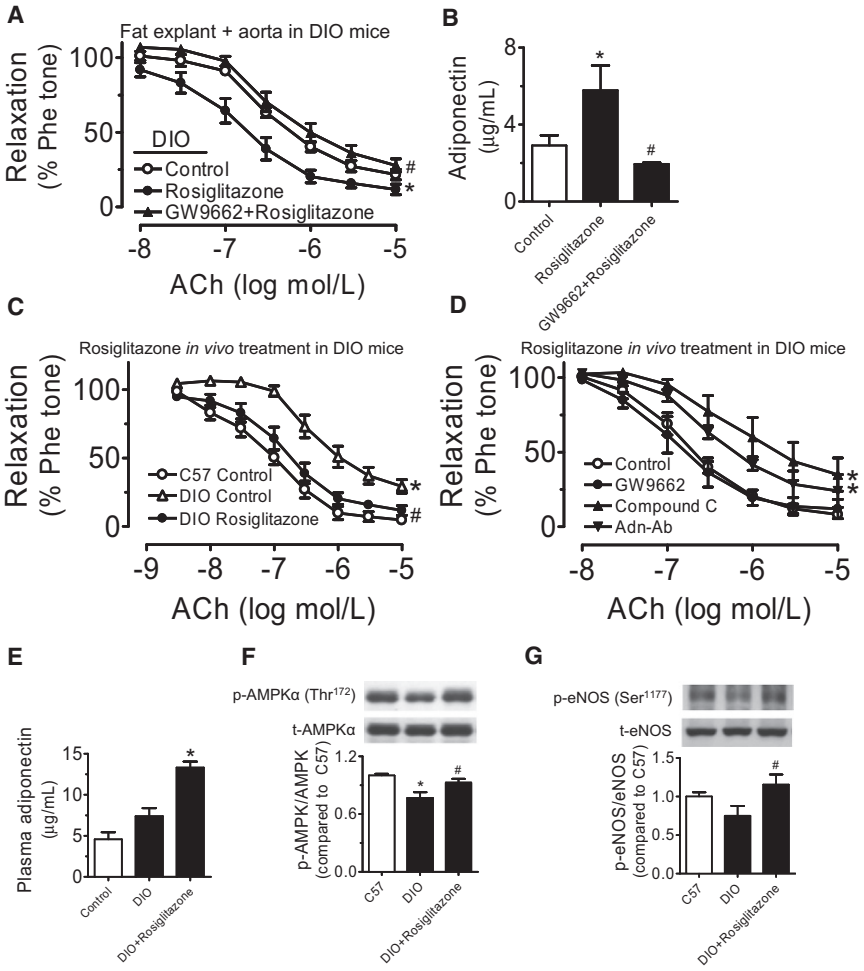

Figure 5. Role of Adiponectin in PPARγ Agonist-Induced Vascular Benefit in DIO Mice

(A) EDRs in aortae from DIO mice improved after treatment in the medium collected from fat explant of DIO mice incubated with rosiglitazone (1 μmol/l, 12 hr) and inhibited by GW9662 (5 μmol/l, 12 hr).

(B) Rosiglitazone (1 μmol/l, 12 hr)-increased adiponectin release in the medium of fat explant and inhibited by GW9662 (5 μmol/l, 12 hr). *p < 0.05 versus control, #p < 0.05 versus rosiglitazone.

(C) Rosiglitazone treatment in vivo improved EDR in aortae from DIO mice. *p < 0.05 versus C57, #p < 0.05 versus DIO control.

(D) Improved EDR in aortae from rosiglitazone-treated DIO mice were inhibited by compound C (5 μmol/l) and anti-adiponectin antibody (Adn-Ab, 5 μg/ml) but unaffected by GW9662 (5 μmol/l). *p < 0.05 versus control.

(E) Rosiglitazone treatment increased plasma adiponectin concentration. *p < 0.05 versus DIO.

(F and G) Western blots showed the increased AMPKα and eNOS phosphorylation in aortae from DIO mice after rosiglitazone treatment. *p < 0.05 versus C57, #p < 0.05 versus DIO. Results are means ± SEM of six mice.

tissue. Although adipose tissue from *db/db* mice expressed less PPARγ and secreted less adiponectin than those from *db/m*$^{+}$ mice, PPARγ activation in subcutaneous fat explants from *db/db* mice shows similar effectiveness in augmenting the EDR in *db/db* mouse aortae. The specificity of PPARγ was further verified by using PPARγ antagonist and aortae from *PPARγ*$^{+/-}$ mice. The obligatory role of adipocyte-derived adiponectin in the vascular benefit of PPARγ agonist is supported by the observation that a neutralizing anti-adiponectin antibody in vitro could eliminate the beneficial effect of PPARγ-activated fat explants and that PPARγ activation in fat explants from *adiponectin*$^{-/-}$ mice failed to improve the EDR of *db/db* mouse aortae.

The benefit of PPARγ activation is further confirmed by the chronic oral administration of rosiglitazone to diabetic mice. Rosiglitazone treatment augmented EDR in aortae from *db/db* mice, improved glucose tolerance, and increased plasma adiponectin level. Since the vascular benefit could be a consequence of systemic improvement of insulin sensitivity in diabetic mice after rosiglitazone treatment, *db/Adn DKO* mice were used to verify the adiponectin-dependent endothelial protection of in vivo PPARγ activation. The potentiating effect of rosiglitazone on endothelial function was blunted in aortae of these animals, indicating an indispensable role of adiponectin in preventing diabetic vascular dysfunction.

To confirm the role of adipose tissue in response to PPARγ activation in diabetic mice, we examined whether or not the benefit of PPARγ activation in adipose tissue on endothelial function can also be transferred. Visceral fat removal or subcutaneous fat transplantation is effective to reverse or prevent insulin resistance and glucose intolerance in diabetic mice (Gabriely et al., 2002; Gavrilova et al., 2000; Tran et al., 2008). The present results showed that fat grafts from rosiglitazone-treated *db/db* mice improved endothelial function after implanting them into untreated *db/db* mice. This benefit lasted in the recipient mice for 2 weeks after the rosiglitazone treatment stopped. This approach strengthened the hypothesis that subcutaneous fat is the major source for the release of adiponectin in response to PPARγ activation in diabetic mice.

TZDs are reported to stimulate adiponectin transcription through a PPAR-responsive element in its promoter in adipocytes and in adipose tissues of obese mice and promote adiponectin secretion from adipocytes (Combs et al., 2002; Iwaki et al., 2003; Maeda et al., 2001; Yang et al., 2004). The present study demonstrates an increased adiponectin release from fat explants upon PPARγ activation. Although the PPARγ expression was less in adipose tissue from *db/db* mice, PPARγ activation in fat explants from these mice showed an effectiveness to release adiponectin similar to fat from nondiabetic mice. A possible explanation is that TZDs improve insulin sensitivity and reverse the proinflammatory changes in adipocytes to facilitate the release of vasoprotective adipokines (Chatterjee et al., 2009; Chui et al., 2005; He et al., 2003; Marchesi et al., 2009; Nawrocki et al., 2006). In addition, we treated DIO mice and found that rosiglitazone treatment produced comparable

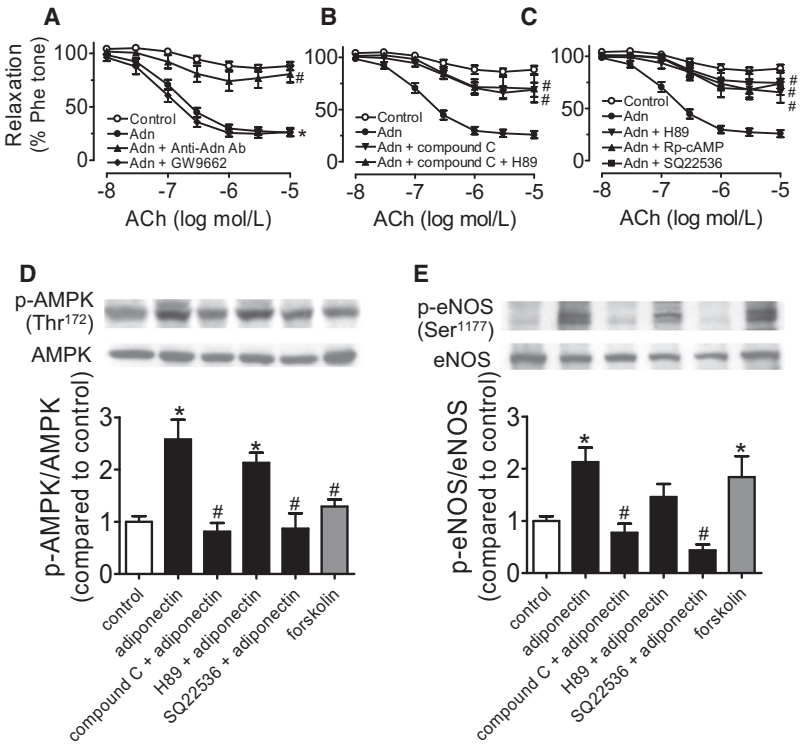

Figure 6. Adiponectin Improves Endothelial Function through AMPK and PKA Signaling

(A) Adiponectin (Adn, 5 μg/ml) alleviated the impaired EDR in *db/db* mouse aortae, and this effect was reversed by anti-adiponectin antibody (Anti-Adn Ab, 5 μg/ml), but unaffected by GW9662 (5 μmol/l).

(B) Effects of compound C (5 μmol/l) and compound C plus H89 (1 μmol/l).

(C) Effects of H89 (1 μmol/l), Rp-cAMP (10 μmol/l), or SQ22536 (100 μmol/l).

(D) Adiponectin (5 μg/ml) increased the phosphorylation of AMPKα at Thr172, which was inhibited by compound C.

(E) Adiponectin (5 μg/ml) increased the phosphorylation of eNOS at Ser1177, which was inhibited by compound C and SQ22536. Results are means ± SEM of 4–6 experiments. *p < 0.05 versus Control, #p < 0.05 versus adiponectin. Control group and *Adn* group in (A)–(C) are from the same data but separated in different graphs for a clearer presentation.

benefits in improving endothelial function and enhancing the release of adiponectin. Like rosiglitazone, two other PPARγ agonists, ciglitazone and troglitazone in the fat explant culture experiments, exhibited similar actions, further supporting the essential role of adiponectin in the improvement of EDR in aortae from both *db/db* and obese mice in response to PPARγ stimulation.

Treatment with TZDs improves cardiovascular outcomes such as hypertension and atherosclerosis (Calkin et al., 2005; Collins et al., 2001; Joner et al., 2007; Ryan et al., 2004; Wang et al., 2004; Yue Tl et al., 2001). In vivo TZD treatment, through adiponectin-dependent mechanisms, reduces pathological revascularizations in the ischemic retina (Higuchi et al., 2010) and inhibits plasminogen activator inhibitor-1 production (Hoo et al., 2007). The present study suggests that the vascular benefit of TZD treatment is largely dependent on adiponectin instead of a systemic improvement of insulin sensitivity, since the glucose tolerance of *db/db* and *db/Adn DKO* mice improved to a similar extent after rosiglitazone treatment, but EDRs were improved by the treatment only in the former. However, direct long-term beneficial effects of TZDs on endothelial cells and vascular smooth muscle cells cannot be excluded. Indeed, several studies suggest that TZDs also act directly on the vasculature to exert anti-inflammatory and antioxidative effects by inhibition of the production of TNF-α and matrix metalloproteinase 9 and of the expression of IκBα (Bishop-Bailey et al., 2002; Chang et al., 2009; de Dios et al., 2003; Goetze et al., 2002; Law et al., 2000; Marx et al., 1998; Orasanu et al., 2008). In type 2 diabetic patients, TZDs also have anti-inflammatory effects by reducing monocyte chemoattractant protein-1, C-reactive protein, and vascular cell adhesion molecules-1 (Ghanim et al., 2006; Hanefeld et al., 2007; Kahn et al., 2010; Kanda et al., 2009; Lombardi et al., 2008; Marx et al., 2003; Mohanty et al., 2004). The present results do not indicate a de novo role for endothelial PPARγ in mediating adiponectin-induced effects, because PPARγ antagonists did not reverse the adiponectin-induced restoration of EDR in aortae from both *db/db* and DIO mice (Figures S4A and S4C) nor affected the improved EDR in aortae from rosiglitazone-treated DIO mice (Figure S4B).

To further investigate the direct effect of adiponectin on vascular function, the effect of full-length mouse recombinant adiponectin was studied. The observations that adiponectin augmented EDR, increased AMPKα and eNOS phosphorylation, and reduced ROS production in aortae from *db/db* mice suggest that both AMPK and cyclic AMP/PKA signaling cascades contribute to the effect of adiponectin in increasing NO bioavailability. The present findings are in line with the observation that AMPK activation is involved in adiponectin-stimulated production of NO in cultured endothelial cells (Chandrasekar et al., 2008; Chen et al., 2003; Cheng et al., 2007; Deng et al., 2010; Ouchi et al., 2004). We demonstrate the functional importance of AMPK activity in adiponectin-induced vascular benefit in intact arteries of diabetic mice.

The adiponectin-induced improvement of EDR can also be mediated by the cyclic AMP/PKA cascade. This conclusion is based on the observation that the responses of *db/db* mouse aortae to adiponectin were inhibited by inhibitors of adenylyl cyclase and PKA. These agents also reduced eNOS phosphorylation. Further experiments demonstrated that adiponectin increased the cyclic AMP content in *db/db* mouse aortae through activation of adenylyl cyclase, independently of AMPK. Importantly, PKA was also involved in the adiponectin-induced ROS reduction in aortae from *db/db* mice. These findings suggest that the vascular effect of adiponectin is also partially mediated through PKA. Previous studies in human endothelial cells showed that cyclic AMP/PKA signaling mediates the inhibitory effect of adiponectin on high-glucose-induced H_2O_2 generation (Ouedraogo et al., 2006). In patients, hypoadiponectinemia

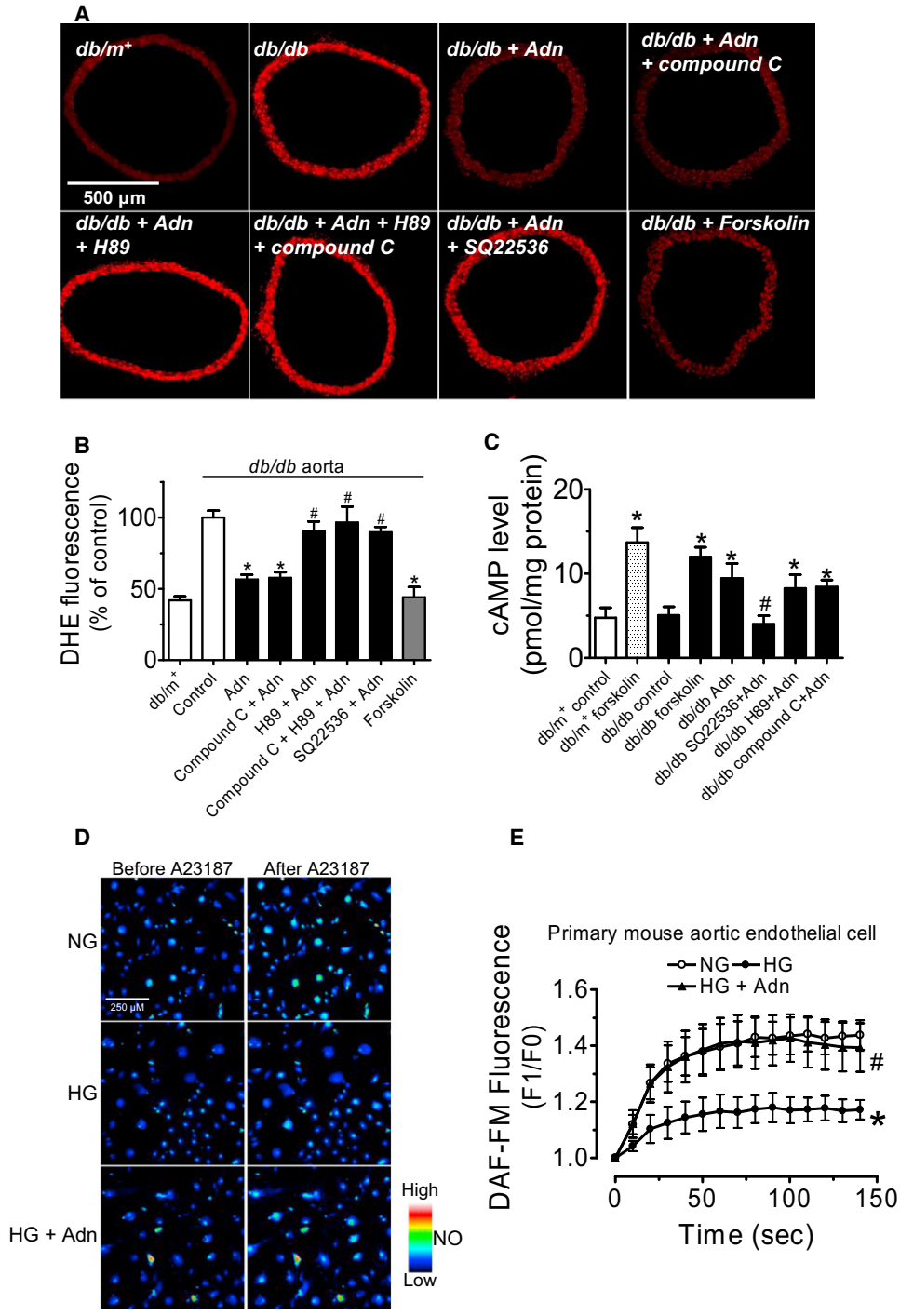

Figure 7. Adiponectin Reduces ROS Generation and Increases NO Bioavailability

(A and B) Representative pictures (A) and summarized data (B) showing adiponectin (5 μg/ml)-reduced ROS accumulation as determined by DHE fluorescence in the vascular wall of aortae from *db/db* mice, and this effect was reversed by H89 or SQ22536, but not by compound C, with forskolin as positive control. (C) Adiponectin (5 μg/ml) increased the production of cyclic AMP in aortae from *db/db* mice, which was inhibited by SQ22536, but not by H89 or compound C. (D and E) Representative images and summarized data showing that adiponectin (5 μg/ml) enhanced the NO production in response to 1 μmol/l A23187 under high-glucose (30 mmol/l, HG) condition. Results are means ± SEM of six experiments. *p < 0.05 versus control within each group, #p < 0.05 versus adiponectin (Adn). NG, normal glucose (5 mmol/l glucose + 25 mmol/l mannitol).

is associated with increased oxidative stress (Lautamäki et al., 2007), suggesting the major involvement of the cyclic AMP/PKA pathway in lowering ROS. However, the possibility cannot be discounted that in vivo TZD treatment results in direct inhibition of oxidative stress, as activation of endothelial PPARγ also exerts anti-inflammatory and antioxidant effects (Beyer et al.,

2008; Ceolotto et al., 2007). Moreover, the antioxidative effect of adiponectin may also be due to the inhibition of NADPH oxidase activity, suppression of IκBα expression, and antagonism of TNF-α (Devaraj et al., 2008; Li et al., 2007; Sharma et al., 2008; Tao et al., 2007; Wang et al., 2009; Zhang et al., 2010).

Several previous reports suggest that adiponectin receptors AdipoR1 and AdipoR2 are expressed in endothelial cells (Cheng et al., 2007; Goldstein and Scalia, 2004; Tan et al., 2004). In bovine endothelial cells, AdipoR1 has higher affinity for globular adiponectin, while AdipoR2 has similar affinity for both globular and full-length adiponectin, which was used in the present experiments (Motoshima et al., 2004). In human endothelial cells, both AdipoR1 and AdipoR2 mediate the effect of adiponectin to stimulate eNOS activity (Cheng et al., 2007). The expressions of both receptors have also been observed in aortae of *db*/*db* mice (Zhang et al., 2010). The present study found that AdipoR2 expression was reduced in aortae from *db*/*db* mice compared with *db*/*m*$^{+}$ mice and increased after rosiglitazone treatment, while AdipoR1 was unaltered. The results indicate additional benefit of PPARγ activation through restoring the reduced AdipoR2 expression in diabetic mouse arteries. The present findings may help to explain that the reduced sensitivity to adiponectin in diabetic mouse arteries (Cheng et al., 2007) may be related to the decreased expression of AdipoR2.

In summary, the present study with focus on this "adipose-vascular loop" demonstrates that PPARγ-activated, adipose tissue-derived adiponectin plays an obligatory role in TZD-induced improvement of endothelial function in diabetes. Adiponectin increases the NO bioavailability by activating AMPK and cyclic AMP/PKA signaling. The present results also support a differential role of various fat depots, which is directly related to the amount of adiponectin released upon PPARγ activation. Adipose tissue could be an important intervention target for newly developed PPARγ agonists in the alleviation of diabetic vasculopathy.

EXPERIMENTAL PROCEDURES

Animals

We used male leptin receptor$^{-/-}$ (*db*/*db*) mice and leptin receptor$^{-/-}$/adiponectin$^{-/-}$ double knockout (*db*/*Adn* DKO) mice (Zhou et al., 2008) and their lean littermates, adiponectin knockout (*Adn*$^{-/-}$) mice (Ma et al., 2002) with a C57BL/6J background and their wild-type controls, and PPARγ heterozygous-deficient (*PPARγ*$^{+/-}$) and wild-type mice (Yu et al., 2008). *PPARγ*$^{+/-}$ mice were used because all homozygous PPARγ knockout animals were embryonically lethal due to placental dysfunction (Yu et al., 2008). The mice were housed in a temperature-controlled holding room (22°C–23°C) with a 12 hr light/dark cycle and fed a diet of standard chow and water. All of the experiments were conducted under the institutional guidelines for the humane treatment of laboratory animals. DIO mice were generated by C57BL/6J, at the age of 6–7 weeks, and were fed with high-fat diet for 12 weeks (rodent diet with 45% kcal% fat, D12451, Research Diets Inc. New Brunswick, NJ).

Ex Vivo Fat Tissue Explant Culture

The method was modified from an established adipose tissue culture technique (Delporte et al., 2002). After the mice were sacrificed, adipose tissues (subcutaneous, visceral, perivascular) were weighted to an equal amount, rinsed in phosphate-buffered saline (PBS), and incubated in Dulbecco's modified Eagle's medium/Ham's F12 medium (HyClone, Ogden, UT). Drugs including rosiglitazone malate (1 μmol/l, GSK No: BRL-49653-C), GW9662 (5 μmol/l), and rabbit polyclonal antibody against mouse adiponectin (5 μg/ml) (Zhou et al., 2008) were added individually. After 12 hr incubation, aliquots of the medium were collected for either assaying adiponectin or incubating aortic rings from *db*/*db* mouse using the same protocol as mentioned above (Figure 1A).

Fat Transplantation

The surgical procedures were approved by the Animal Experimental Ethics Committee, CUHK. Methods were modified from several groups (Gabriely et al., 2002; Gavrilova et al., 2000; Tran et al., 2008). To avoid rejection, donor fat grafts were taken from *db*/*db* littermates. Mice were anaesthetized with a mixture of 35 mg/kg ketamine and 7 mg/kg xylazine. Fat transplantation was performed using fat pads removed from the subcutaneous dorsal area. Fat pads were removed, cut into approximately 200 mg pieces, and kept in saline warmed at 37°C until transplantation. For each recipient mouse, a total of 1.0 g subcutaneous fat was removed from the dorsal area; similarly sized donor slices of fat were transplanted into the dorsal area. All mice received subcutaneous injection of penicillin and streptomycin after surgery and were housed in individual cages for 2 weeks before sacrifice. After sacrifice, fat grafts were examined to detect whether there was necrosis, which was taken as unsuccessful surgery.

Statistics

Results represent means ± SEM from different mice. Concentration-response curves were analyzed by nonlinear regression curve fitting using GraphPad Prism software (Version 4.0) Comparisons among groups were made using ANOVA followed by an unpaired Student's t test. The p values less than 0.05 were accepted to indicate statistically significant differences.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, and four figures and can be found with this article online at doi:10.1016/j.cmet.2011.05.009.

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