Dynamic Changes of Adiponectin and S100A8 Levels by the Selective Peroxisome Proliferator–Activated Receptor-γ Agonist Rivoglitazone

Aki Hiuge-Shimizu, Norikazu Maeda, Ayumu Hirata, Hideaki Nakatsuji, Kouichi Nakamura, Akira Okuno, Shinji Kihara, Tohru Funahashi, Iichiro Shimomura

Objective—Accumulating evidence indicates that the regimen to increase adiponectin will provide a novel therapeutic strategy for metabolic syndrome. Here, we tested the effect of a potent and selective peroxisome proliferator–activated receptor-γ agonist, rivoglitazone (Rivo), a newly synthesized thiazolidinedione derivative, on adiponectin, insulin resistance, and atherosclerosis.

Methods and Results—ob/ob mice, apolipoprotein E knockout (apoE KO) mice, and apoE and adiponectin double knockout mice were administered pioglitazone, Rivo, or no compound. Remarkable elevation of plasma adiponectin was observed, especially in Rivo-treated ob/ob mice. Rivo ameliorated insulin resistance in ob/ob mice and reduced atherosclerotic areas in apoE KO mice compared with the pioglitazone group but failed to decrease atherosclerotic areas in double knockout mice. Among adipose mRNAs, adipose S100A8, which activates Toll-like receptor 4–dependent signal transduction cascades and locates upstream of inflammation, was markedly increased in ob/ob mice, and its increase was completely reversed by Rivo treatment. In RAW264.7 macrophage cells and 3T3-L1 adipocytes, Rivo significantly reduced S100A8 mRNA levels.

Conclusion—The peroxisome proliferator–activated receptor-γ agonist Rivo remarkably enhanced adiponectin in plasma and decreased adipose S100A8 mRNA levels in obese mice. Rivo treatment apparently ameliorated insulin resistance in ob/ob mice and reduced atherosclerosis in apoE KO mice, partly through adiponectin.

Key Words: adiponectin ■ inflammation ■ oxidative stress ■ peroxisome proliferator-activated receptor ■ S100A8
mice were fed a high-fat atherogenic Western diet (Oriental Yeast; 20% fat, 0.15% cholesterol) containing 0.01% Pio, 0.003% Rivo (Rivo-H), or no compound (Cont) from 5 weeks of age. At 10 weeks of age, mice were anesthetized and analyzed. Perfusion-fixation, preparation of aortas, and quantification of atherosclerotic lesions were performed. Lesions in the proximal aorta from serial 10-μm-thick cryosections were stained with Oil Red O, counterstained with hematoxylin, and quantified with an imaging computer software, and the mean values were determined as previously described.8

WAT from male C57BL/6J mice were fractionated. Adipose tissues were minced in Krebs-Ringer buffer, which contained 120 mM/L NaCl, 4 mM/L KH₂PO₄, 1 mM/L MgSO₄, 1 mM/L CaCl₂, 10 mM/L NaHCO₃, 30 mM/L HEPES, 20 μM/L adenosine, and 4% (wt/vol) bovine serum albumin (Calbiochem, San Diego, CA). Tissue suspensions were centrifuged at 500 g for 5 minutes to remove erythrocytes and free leukocytes. Collagenase was added to a final concentration of 2 mg/mL and incubated at 37°C for 20 minutes with shaking. The cell suspension was filtered through a 250 μm filter and then spun at 300g for 1 minute to separate floating mature adipocytes fraction from the stromal vascular cell (SVC) fraction pellet. This fractioning and washing procedure were repeated twice with Krebs-Ringer buffer. Finally, both fractions were washed with phosphate-buffered saline. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Cell Cultures
3T3-L1 cells were maintained and differentiated as previously described.11 On day 4, 3T3-L1 adipocytes were treated with the indicated concentrations of either Pio or Rivo dissolved in dimethyl sulfoxide for 24 hours. An aliquot of the medium was subjected to measurement of adipogenic protein by using an ELISA kit (Otsuka, Tokushima, Japan). For the adipogenic promoter analysis, we used the Renilla luciferase (pRL)-SV40 (internal standard) with pGL3-plasmid containing the 5′-flanking region of the human adiponectin gene (−908 to −flanking region of the human adiponectin gene (−908 to +14) (Adn-pGL3 [wild-type]), the point mutation in PPAR-responsive element (PPRE) site of human adiponectin gene (Adn-pGL3 [PPREM]), or pGL3-basic alone.1.13

On day 4 after differentiation, the media of 3T3-L1 cells in 12-well plates were changed to Opti-MEM (Life Technologies), and cells were transfected with the indicated plasmids (10 ng/well of pGL3-basic plasmid) by using 6 μl/well of Lipofectamine 2000 reagent (Life Technologies). Three hours later, an equal amount of Dulbecco’s modified Eagle’s medium containing Pio and Rivo was added to the media. At 48 hours after transfection, luciferase reporter activities were measured by the Dual-Luciferase Reporter Assay System (Promega), and the transfection efficiencies were normalized to the Renilla luciferase activity.

RAW264.7 macrophage cells were obtained from ATCC (TIB-71) and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin and streptomycin. The RAW264.7 cells were plated at a density of 2.5×10⁵ cells/well in 12-well plates maintained at 37°C in a humidified atmosphere containing 5% CO₂ for 24 hours. Cells were treated with the indicated concentrations of Rivo for 48 hours and harvested.

Quantification of mRNA Levels
Total RNA was isolated from mice tissues by using RNA STAT-60 (Tel-Test Inc., Friendswood, TX) according to the protocol supplied by the manufacturer. The quality and quantity of total RNA was determined by using ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE). First-strand cDNA was synthesized from 180 ng of total RNA using Thermoscript RT (Invitrogen Corp., Carlsbad, CA) and oligo(dt) primer. Real-time quantitative polymerase chain reaction amplification was conducted with the LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan) using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the protocol recommended by the manufacturer. Primer sets were as follows: mouse adiponectin, 5′-GGATGCCAGAGATTGCAC TCC-3′ and 5′-CTCGACATGCTGCCTCT-3′; mouse NADPH oxidase p2phox, 5′-CTGCGCTAGCCGAGATG-3′ and 5′-CAATGGGCACAGCGGTC-3′; mouse NADPH oxidase p67phox, 5′-CTGCGCTAGCCGAGATG-3′ and 5′-CAATGGGCACAGCGGTC-3′; mouse adiponectin, 5′-AGCGCGGTTCCTGGCATTGTCT-3′ and 5′-CACCGTGCCA CACGCTCT-3′; mouse catalase, 5′-CCACGGAACAGATTGAAGC CT-3′ and 5′-CCACCGGGAATGCAGACGGTC-3′; mouse TNF-α, 5′-GCCACACCGCCTTCTG-3′ and 5′-GGTGGTGGTGAGCAACA-3′; mouse monocyte chemoattractant protein-1 (MCP-1), 5′-CCACTCAC TCGTGGTGAATGCTGTCC-3′; mouse glutathione S-transferase A4 (GSTA4), 5′-CGATGTTGGATGCTGACACA-3′ and 5′-CAGTGGAAGAATACGGGTGTACGC-3′; mouse 100A8, 5′-GCCACCCAGCCTCTGCTCT-3′ and 5′-GGTGTGGTGAGAGCAACA-3′.

The final result for each sample was normalized to the respective 36B4 value.

Statistical Analysis for the In Vivo and In Vitro Experiments
Results were expressed as the mean±SEM of n separate experiments. Differences between groups were examined for statistical significance using the Student t test or ANOVA with the Fisher protected least significant difference test. A probability value less than 0.05 indicated a statistically significant difference.

Results
Effect of Rivo on Obese Model Mice
Firstly, ob/ob mice and lean Cont C57BL/6J mice were fed a regular diet containing 0.01% Pio, Rivo-L, or no compound (Cont) from 22 to 25 weeks of age. Mice were given an insulin tolerance test at day 14 from treatment (Figure 1A). Plasma glucose levels of Rivo-H–treated C57BL/6J mice were significantly lower than those of the Cont group at 60 minutes (P<0.05). In ob/ob mice, the initial plasma glucose levels were significantly decreased in Pio-, Rivo-L-, and Rivo-H–treated mice compared with Cont group (P<0.05, P<0.001, and P<0.001, respectively). Plasma glucose levels of Rivo-L–treated mice were significantly lower than those of Pio-treated mice at 30 and 60 minutes. Moreover, Rivo-H–treated ob/ob mice exhibited lower glucose levels than Pio-treated mice at 15, 30, 60, and 90 minutes after insulin injection, when concentrations of these compounds were similar in plasma (Pio: 2.569±0.162 μmol/L, Rivo-H: 2.364±0.145 μmol/L). When plasma glucose levels for each time point after insulin injection versus preinjection were analyzed in ob/ob mice, there was no significant difference of plasma glucose at each time point compared with preinjection in Pio-treated mice. In Rivo-L–treated obese mice, plasma glucose levels at 60 and 90 minutes were significantly lower than those at 0 minutes.

In Rivo-H–treated ob/ob mice, the plasma glucose level at 60 minutes was significantly lower than that at 0 minutes. Figure 1B shows the area under the curve of plasma glucose during the insulin tolerance test. TZD treatments apparently decreased areas under the curve in ob/ob mice; besides, areas under the curve of Rivo-L– and Rivo-H–treated mice were significantly lower than those of Pio-treated mice. Rivo treatment ameliorated plasma lipid profiles (Figure 1C). Especially, total cholesterol, triglyceride, and free fatty acids (FFA) in plasma were significantly decreased in Rivo–treated ob/ob mice.
Adiponectin levels were also examined in C57BL/6J mice and ob/ob mice at day 21 after treatment (Figure 2). Plasma adiponectin levels were significantly elevated in Pio-, Rivo-L-, and Rivo-H–treated C57BL/6J mice (Figure 2A; plasma adiponectin levels: 22.3±4.9 μg/mL in Cont, 33.4±4.3 μg/mL in Pio, 65.1±11.0 μg/mL in Rivo-L, and 79.2±9.0 μg/mL in Rivo-H). In ob/ob mice, plasma adiponectin levels were also increased by TZD treatment (Figure 2A; plasma adiponectin levels: 17.4±0.9 μg/mL in Cont, 37.2±8.1 μg/mL in Pio, 83.0±9.4 μg/mL in Rivo-L, and 196.8±32.3 μg/mL in Rivo-H). As shown in Figure 2A, plasma adiponectin levels were remarkably elevated in Rivo-H–treated ob/ob mice. Adiponectin mRNA levels of WAT were also enhanced in TZD-treated C57BL/6J mice (Figure 2B). In the Cont group, adiponectin mRNA levels were lower in ob/ob mice compared with C57BL/6J mice. TZDs also increased mRNA levels of adiponectin in ob/ob mice. Curiously, in Rivo-H-treated ob/ob mice, the induction levels of plasma adiponectin (Rivo-L, 4.46±0.21-fold increase from Cont; Rivo-H, 11.31±0.76-fold increase from Cont) were markedly higher than the increased levels of adiponectin mRNA (Rivo-L, 1.62±0.11-fold increase from Cont; Rivo-H, 1.99±0.15-fold increase from Cont).

**Effect of Rivo on Atherosclerosis Model Mice**

We next examined the effect of Rivo on atherosclerosis by using apoE KO mice under an atherogenic Western diet. Pio tended to reduce atherosclerotic area, and Rivo-H significantly decreased atherosclerotic area. Significant increases in plasma adiponectin were also observed in Pio- and Rivo-H–treated apoE KO mice (Figure 3C). ApoE KO mice were sequentially crossed with adiponectin-null mice, and thus DKO mice were generated to investigate whether TZD-mediated elevation of adiponectin plays a protective role on atherosclerosis. Atherosclerotic areas were not significantly reduced by Pio and Rivo-H treatment in DKO mice under an atherogenic Western diet (Supplemental Figure I, available online at http://atvb.ahajournals.org), indicating that TZD-mediated increase of plasma adiponectin plays a protective role in atherosclerosis in this animal model.

**Effect of Rivo on Adiponectin in 3T3-L1 Adipocytes**

Next, we examined the effect of Rivo on adiponectin by using 3T3-L1 adipocytes (Figure 4). Adiponectin mRNA and protein levels were significantly increased by Pio and Rivo treatment, but there were no significant differences in these parameters between Pio and Rivo (Figure 4A and 4B). TZDs increase adiponectin at the transcriptional level through the peroxisome proliferators response element (PPRE) located on its promoter, as demonstrated previously. Figure 4C shows activities of adiponectin promoter by using 3T3-L1 adipocytes.
pocytes. Pio significantly enhanced adiponectin promoter activity (Figure 4C, lane 2 versus lane 3), and Rivo also elevated its activity in a dose-dependent manner (lane 4 versus lanes 5 to 9). Pio- and Rivo-induced activations of adiponectin promoter were completely abolished when the point mutation was inserted in PPRE site (Figure 4C, lane 3 versus lane 10, and lane 8 versus lane 11).

Effect of Rivo on Adipose mRNA Levels Relating to ROS and Cytokines in Obese Model Mice

To explore the mechanism of marked induction of plasma adiponectin in ob/ob mice as shown in Figure 2A, the mRNA levels relating to ROS and cytokines were examined in WAT (Figure 5). NADPH oxidase, which is a ROS-generating enzyme, subunit p22 and p67 mRNA levels were higher in

![Figure 3](image-url)

**Figure 3.** Effect of Rivo on atherosclerosis model mice. ApoE KO mice were fed an atherogenic Western diet containing 0.01% Pio, Rivo-H, or no compound (Cont), from 5 to 10 weeks of age. A, Sections of aortic sinus stained by Oil Red O. Scale bar=100 μm. B, Quantification of atherosclerotic area after the indicated treatment. C, Plasma adiponectin concentrations. Values are mean±SEM; n=6 for each group. *P<0.05, ***P<0.001, compared with the values of the Cont group.

![Figure 4](image-url)

**Figure 4.** Effect of Rivo on adiponectin in 3T3-L1 adipocytes. A, Adiponectin mRNA levels. B, Adiponectin protein levels in media. C, Adiponectin promoter activity. Adn-pGL3 (wt) indicates pGL3-basic plasmid containing the S'-flanking region of human adiponectin gene (−908 to +14); Adn-pGL3 (PPREmut), pGL3-basic plasmid containing the point mutation in PPRE site of human adiponectin gene (−908 to +14). Adiponectin-luciferase activity was normalized by pRL-SV40 Renilla luciferase activity and represented in fold induction compared with the left lane. Values are mean±SEM; n=6 for each treatment. *P<0.05, **P<0.01, ***P<0.001, compared with the values of the untreated group (A and B). ***P<0.001, compared with the values of lane 2; †††P<0.001, compared with the values of lane 4; ####P<0.001, compared with values of lane 3 vs lane 10 or lane 8 vs lane 11 respectively (C).
apparent differences between Pio- and Rivo-treated mice demonstrated in Figure 5A to 5D, but there were no differences when compared with the values of the Cont group (Figure 5E). However, TNF-α and MCP-1 mRNA levels were elevated in ob/ob mice, whereas adiponectin was expressed only in mature adipocytes fraction (data not shown).

Adipose expression of S100A8 and the response of S100A8 by Rivo in 3T3-L1 adipocytes and RAW264.7 cells

The expression level of S100A8 has not been investigated in fat tissues, and thus tissue distribution of S100A8 was examined in WAT. Adipose S100A8 mRNA levels were examined after 3 weeks of indicated treatment as shown in Figure 1. Values are mean ± SEM; n = 6 for each group. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the values of the Cont group.

Figure 5. Effect of Rivo on mRNA levels in WAT. Adipose mRNA levels were examined 3 weeks after induction for S100A8 and the response of S100A8 by Rivo in 3T3-L1 adipocytes and RAW264.7 cells.
pocytes, Rivo strongly decreased S100A8 and MCP-1 mRNA levels (Figure 6E and 6F).

Discussion

In this study, we demonstrated that (1) Rivo treatment significantly ameliorated insulin resistance and atherosclerosis in ob/ob and apoE KO mice, respectively; (2) TZDs failed to reduce atherosclerosis areas in DKO mice; (3) remarkable elevation of plasma adiponectin was observed in Rivo-H–treated ob/ob mice; (4) Rivo attenuated adipose dysregulation of GSTA4 and S100A8 mRNA levels in ob/ob mice; and (5) S100A8 mRNA was expressed in both mature adipocytes and SVCs of WAT and was significantly reduced by Rivo in both 3T3-L1 adipocytes and RAW264.7 macrophage cells.

Plasma triglyceride levels of Rivo-H–treated ob/ob mice were apparently decreased compared with those of Pio-treated obese mice. PPARγ agonists decreased hypertrophic adipocytes, whereas such treatment increased small adipocytes in WAT as demonstrated previously.19 Histological analysis of obese WAT was also performed in the present study, and TZDs actually increased the number of small adipocytes (Supplemental Figure III). The number of small adipocytes tended to increase in Rivo-H–treated ob/ob mice compared with Pio-treated ob/ob mice. The amounts of FFA secretion are lower in small adipocytes than in hypertrophic adipocytes, suggesting that Rivo treatment may reduce FFA level partly through the increased number of small adipocytes. The decrease of FFA attenuates insulin resistance, resulting in the promotion of very-low-density lipoprotein catabolism. Finally, plasma triglyceride level may be decreased by PPARγ agonists.20 Furthermore, a remarkable elevation of plasma adiponectin was observed in Rivo-H–treated obese mice as shown in Figure 2A. Adiponectin transgenic mice resulted in the reduction of very-low-density lipoprotein fraction in the low-density lipoprotein receptor-null background.21 Collectively, there is a possibility that Rivo-H treatment reduces very-low-density lipoprotein level partly through the dynamic elevation of adiponectin in obese mice, but further investigation will be required to clarify the effect of Rivo on lipoprotein metabolism.

Insulin resistance and atherosclerosis are closely associated with chronic low-grade inflammation, which occurs not only locally in WAT and vasculature but also systemically.22,23 In obese adipose tissue, adipocytes and infiltrated macrophages communicate with each other through fatty acids and cytokines. Such interactions cause adipocytokine dysregulation.
and finally develop into insulin resistance and atherosclerosis. Our group previously demonstrated that ROS is significantly increased in obese fat tissue. ROS and inflammatory signaling interact each other and form a vicious cycle in various tissues. On the other hand, TZDs possess a beneficial action against ROS, inflammation, and adipocytokine dysregulation. We here newly found out the TZD-mediated regulations of S100A8 and GSTA4 in vivo during the analysis of mechanism for the marked elevation of plasma adiponectin in Rivo-H–treated ob/ob mice. In addition, Rivo-H treatment reduced atherosclerotic areas in apoE KO mice, but not in DKO mice, suggesting that TZD-mediated elevation of plasma adiponectin may protect against atherosclerosis. Rivo also elevated the plasma adiponectin level in subjects with type 2 diabetes, but the effect of Rivo on atherosclerosis remained uncertain in human subjects.

ROS accelerates peroxidation of polyunsaturated fatty acids and generates a family of reactive α,β-unsaturated aldehydes, such as trans-4-hydroxy-2-nonenal. Reactive lipid aldehydes covalently modify protein, generically termed protein carbonylation, resulting in dysfunction of the corresponding protein. GSTA4 efficiently catalyzes the conjugation of trans-4-hydroxy-2-nonenal to glutathione, leading to their efflux from the cell via glutathione conjugate transporter RLIP76. Importantly, Curtis et al recently reported that adipose GSTA4 was decreased in obesity and insulin resistance, suggesting a possibility that some overcarbonylated protein may be related to the decreased secretion of adiponectin in obese fat tissue. The present study showed for the first time that GSTA4 was significantly enhanced by TZDs, especially by Rivo (Figure 4E), indicating that TZD-mediated PPARγ activation may reduce the excess of ROS exposure and normalize intracellular protein function in obese adipocytes. GSTA4 mRNA was dominantly detected in mature adipocytes fraction compared with SVs fraction (data not shown). We also examined the effect of the TZDs Pio and Rivo on GSTA4 mRNA levels in 3T3-L1 adipocytes, but the elevation of GSTA4 mRNA was not observed in vitro (data not shown). A similar result was previously demonstrated when GSTA3, a member of GSTs, was transcriptionally enhanced by 15-deoxy-Δ12,14-prostaglandin J2, known to be an endogenous PPARγ ligand, through an antioxidant response element, and rosiglitazone failed to elevate the promoter activity of GSTA3. Moreover, there was no putative PPRE site on the human GSTA4 promoter. These results suggest that TZDs may indirectly elevate GSTA4 mRNA levels in vivo adipose tissue, but the precise mechanism should be clarified in the future.

S100A8 (also termed myeloid-related protein-8) belongs to the family of S100 proteins and forms a homodimer or a heterodimer with S100A9 (also termed myeloid-related protein-14). The complex of S100A8 and S100A9 (also called calprotectin) is actively secreted during the stress response of phagocytes. S100A8 binds to Toll-like receptor 4 and activates Toll-like receptor 4–dependent signal transduction cascades. In addition, S100A8/S100A9 is associated with the receptor for advanced glycation end products and elevated the secretion and gene expressions of proinflammatory cytokines in human umbilical vein endothelial cells under the receptor for advanced glycation end products. Increasing evidence shows that S100A8/S100A9 plays a crucial role in inflammation, autoimmunity, cancer, and atherosclerosis. The present study shows for the first time that WAT is a major source of S100A8 (Figure 6A) and that adipose S100A8 is significantly elevated in obese model mice (Figure 6H). Furthermore, PPARγ agonists ameliorated the increase of S100A8 mRNA levels of obese mice, and especially, Rivo completely reversed its mRNA level, indicating that S100A8 may be one of the genes transrepressed by PPARγ agonists. PPARγ ligands induce sumoylation of PPARγ by small ubiquitin-like modifier 1 and sumoylated PPARγ binds to the nuclear receptor corepressor complexes, resulting in the inhibition of signal-dependent nuclear receptor corepressor turnover. Consequently, nuclear receptor corepressor complexes continue to repress transcription of inflammatory-responsive genes. Taken together, PPARγ agonists may cut off the vicious inflammatory cycle in obese adipose tissue partly through the repression of S100A8. However, further investigation will be required to clarify the molecular mechanism for the effect of PPARγ on S100A8. In addition, there is a possibility that Rivo-mediated amelioration of S100A8 may reflect one of the mechanisms for the remarkable induction of plasma adiponectin level by Rivo. The association of adiponectin and S100A8 should warrant further investigation.

In summary, a potent and selective PPARγ agonist, Rivo, apparently ameliorated insulin resistance and atherosclerosis, and it remarkably enhanced plasma adiponectin level in obese mice. S100A8 is one of the genes repressed by PPARγ agonist.

Acknowledgments
We thank Yoko Motomura and Mina Sonoda for the excellent technical assistance in 3T3-L1 culture.

Sources of Funding
This work was supported in part by Grants-in-Aid for Scientific Research B 19390249 (to T.F.) and C 22590979 (to N.M.).

Disclosures
Drs Nakamura and Okuno are employed by Daiichi Sankyo Co, Ltd. and declare no conflict of interest.

References


Dynamic Changes of Adiponectin and S100A8 Levels by the Selective Peroxisome Proliferator–Activated Receptor-γ Agonist Rivoglitazone

Aki Hiuge-Shimizu, Norikazu Maeda, Ayumu Hirata, Hideaki Nakatsuji, Kouichi Nakamura, Akira Okuno, Shinji Kihara, Tohru Funahashi and Iichiro Shimomura

Arterioscler Thromb Vasc Biol. 2011;31:792-799; originally published online January 13, 2011; doi: 10.1161/ATVBAHA.110.221747

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/31/4/792

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/01/13/ATVBAHA.110.221747.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Figure Legends

Supplemental Figure I.
Effect of TZDs on apolipoprotein E-deficient and adiponectin-deficient (DKO) mice.
DKO mice were fed with atherogenic western diet, containing either 0.01% pioglitazone (Pio), 0.003% rivoglitazone (Rivo-H), or no compound (Cont), from 5 to 10 weeks of age. A, Sections of aortic sinus stained by oil red O. Scale bar = 100 µm. B, Quantification of atherosclerotic area after the indicated treatment. Values are mean ± SEM; n=6 for each group. NS indicates not significant.

Supplemental Figure II.
Effect of rivoglitazone on adipose ROS in obese mice. Thiobarbituric acid reactive substances (TBARS) levels were examined in WAT of ob/ob mice treated with 0.01% pioglitazone (Pio), 0.0005% rivoglitazone (Rivo-L), 0.003% rivoglitazone (Rivo-H), or no compound (Cont) from 22 to 25 weeks of age. Values are mean ± SEM; n=6 for each group. *P<0.05, compared with the values of Cont group.

Supplemental Figure III.
Cell size distribution of obese WAT. The isolated adipose tissue of ob/ob mice was formalin-fixed, paraffin-embedded, and subsequently cut into 6 µm sections and mounted on glass slides using standard procedures. The sections were stained with haematoxylin and eosin. The area of adipocytes was traced manually and measured in
≥200 cells per mouse by using Win ROOF 5.5 software (Mitani Co, Fukui, Japan).
Cont, no compound; Pio, 0.01% pioglitazone; Rivo-H, 0.003% rivoglitazone.
Supplemental Figure 1

(A) Representative micrographs of atherosclerotic plaques in DKO + Cont, DKO + Pio, and DKO + Rivo-H.

(B) Bar graph showing the atherosclerotic area (mm²) in each group. The graph indicates no significant difference (NS) between the groups.

Legend:
- Cont
- Pio
- Rivo-H

Atherosclerotic area (mm²)
Supplemental Figure III