

# Peroxisome Proliferator–Activated Receptor (PPAR) $\alpha$ Activation Increases Adiponectin Receptors and Reduces Obesity-Related Inflammation in Adipose Tissue

## Comparison of Activation of PPAR $\alpha$ , PPAR $\gamma$ , and Their Combination

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We examined the effects of activation of peroxisome proliferator–activated receptor (PPAR) $\alpha$ , PPAR $\gamma$ , and both of them in combination in obese diabetic KKAY mice and investigated the mechanisms by which they improve insulin sensitivity. PPAR $\alpha$  activation by its agonist, Wy-14,643, as well as PPAR $\gamma$  activation by its agonist, rosiglitazone, markedly improved insulin sensitivity. Interestingly, dual activation of PPAR $\alpha$  and  $\gamma$  by a combination of Wy-14,643 and rosiglitazone showed increased efficacy. Adipocyte size in Wy-14,643–treated KKAY mice was much smaller than that of vehicle- or rosiglitazone-treated mice, suggesting that activation of PPAR $\alpha$  prevents adipocyte hypertrophy. Moreover, Wy-14,643 treatment reduced inflammation and the expression of macrophage-specific genes in white adipose tissue (WAT). Importantly, Wy-14,643 treatment up-regulated expression of the adiponectin receptor (AdipoR)-1 and AdipoR2 in WAT, which was decreased in WAT of KKAY mice compared with that in nondiabetic control mice. Furthermore, Wy-14,643 directly increased expression of AdipoRs and decreased monocyte chemoattractant protein-1 expression in adipocytes and macrophages. Rosiglitazone increased serum adiponectin concentrations and the ratio of high molecular weight multimers of adiponectin to total adiponectin. A combination of rosiglitazone and Wy-14,643 increased both serum adiponectin concentrations and AdipoR expression in WAT. These data suggest that PPAR $\alpha$  activation prevents inflammation in WAT and that dual activation of PPAR $\alpha$  and  $\gamma$  enhances the action of adiponectin by increasing both adiponectin and AdipoRs,

which can result in the amelioration of obesity-induced insulin resistance. *Diabetes* 54:3358–3370, 2005

**P**eroxisome proliferator–activated receptor (PPAR) $\alpha$  and  $\gamma$  are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily that regulate the metabolism of glucose and lipids (1–6). PPAR $\gamma$  is one of the key regulators of glucose homeostasis, and the molecular mechanisms concerning how the activation of PPAR $\gamma$  improves insulin sensitivity have been well investigated. Activation of PPAR $\gamma$  by agonists such as thiazolidinediones (TZDs) stimulates lipid storage in adipocytes, thereby reducing lipotoxicity in liver and skeletal muscle (7). In addition, PPAR $\gamma$  activation increases small adipocytes, thereby increasing the insulin-sensitizing hormone adiponectin and reducing resistin and tumor necrosis factor (TNF)- $\alpha$ , which induce insulin resistance (8,9). However, PPAR $\gamma$  agonists are associated with body weight gain, which is a clinical drawback for treatment of type 2 diabetic patients. In contrast, it has been previously reported that PPAR $\alpha$  agonists prevent the development of obesity-induced insulin resistance in rodents without inducing body weight gain (10–12); however, the mechanisms by which the activation of PPAR $\alpha$  improves insulin resistance are not fully understood.

Recently, it has been reported that chronic inflammation in white adipose tissue (WAT) by macrophage infiltration may cause whole-body insulin resistance in obese diabetic animals (13,14). Activated macrophages that infiltrate into WAT secrete cytokines that can impair adipocyte insulin sensitivity. Adipocytes stimulated by proinflammatory cytokines secrete chemokines that can contribute to macrophage infiltration. This vicious cycle impairs adipocyte insulin signaling and may eventually cause systemic insulin resistance (13,14). Moreover, inflammatory markers such as C-reactive protein are associated with insulin resistance, adiposity, and type 2 diabetes in human subjects (15–17). Therefore, it has become important to investigate the mechanisms of insulin-sensitizing drugs by focusing on the regulation of inflammation.

In this study, we examined the effects of activation of

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AdipoR, adiponectin receptor; BAT, brown adipose tissue; DMEMH, Dulbecco's modified high-glucose Eagle's medium; HMW, high molecular weight; MCP, monocyte chemoattractant protein; PDK4, pyruvate dehydrogenase kinase isozyme 4; PPAR, peroxisome proliferator–activated receptor; TNF, tumor necrosis factor; TZD, thiazolidinedione; UCP, uncoupling protein; WAT, white adipose tissue.

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PPAR $\alpha$ , PPAR $\gamma$ , and both in combination in obese diabetic KKAY mice, and we investigated the mechanisms by which they improve insulin resistance, especially in WAT. The results indicate that PPAR $\alpha$  activation prevents infiltration of macrophages into WAT, thereby ameliorating inflammation of WAT, which can result in improvement of obesity-induced insulin resistance. We also demonstrate that dual activation of PPAR $\alpha$  and  $\gamma$  enhances the action of adiponectin in WAT by increasing both adiponectin and the expression of its receptors.

## RESEARCH DESIGN AND METHODS

Rosiglitazone was synthesized as described elsewhere (18). Wy-14,643 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). All materials were obtained from sources described previously (8,9,19).

We purchased 6-week-old male KKAY mice and age-matched KK mice from Nippon CLEA (Shizuoka, Japan). KKAY mice are an obese diabetic model in which the Ay mutation is introduced onto a KK strain background. Therefore, we used KK mice as nondiabetic controls. Mice were housed singly and maintained on a 12-h light/dark cycle. The high-fat diet consisted of 32% (wt/wt) fat as described previously (20). KKAY mice were given the high-fat diet, and KK mice were given normal chow. High-fat feeding was begun 1 week before rosiglitazone or Wy-14,643 administration. Rosiglitazone or Wy-14,643 was given as a 0.01 and 0.05% food admixture, respectively. These doses were chosen because they have been shown to be effective therapeutic doses in diabetic mice (21–24). The same amount of food was given to the pair-fed group of KKAY mice as that fed to the Wy-14,643-treated group of KKAY mice. The animal care procedures and methods were approved by the animal care committee of the University of Tokyo.

**Blood sample assays and in vivo glucose homeostasis.** The glucose tolerance and insulin tolerance tests were carried out according to previously described methods (9). Plasma glucose, serum free fatty acid, and triglyceride levels were determined by a glucose test, nonesterified fatty acid-C test, and triglyceride L-type (Wako Pure Chemical Industries, Osaka, Japan), respectively. Plasma insulin was measured by an insulin immunoassay (Shibayagi, Gunma, Japan). Plasma leptin and adiponectin levels were determined by a Quintikine M kit (R&D Systems, Minneapolis, MN) and mouse adiponectin immunoassay kit (Otsuka Pharmaceutical, Tokushima, Japan), respectively (20). Detection of multimer species of adiponectin was conducted as described previously (25). In brief, 0.7  $\mu$ l of serum was subjected to 2–15% SDS-PAGE under nonreducing and non-heat-denaturing conditions. Adiponectin was detected using anti-globular domain antiserum obtained by immunizing rabbits with mouse recombinant adiponectin globular domain produced in *Escherichia coli* (25).

**Histological analysis of adipose tissue.** Epididymal adipose tissue was removed from each animal, fixed in 10% formaldehyde/PBS, and maintained at 4°C until use. Fixed specimens were dehydrated, embedded in tissue-freezing medium (Tissue-Tek OCT compound; Miles, Elkhart, IN), and frozen in dry ice and acetone. WAT was cut into 10- $\mu$ m sections, and the sections were mounted on silanized slides. The adipose tissue was stained with hematoxylin and eosin (7) or anti-mouse F4/80 antibody (Serotec, Raleigh, NC) (13).

**Quantitative analysis by real-time PCR.** Total RNA was prepared from cells or tissues with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A real-time PCR method was used to quantify the AdipoRs mRNAs (19). The primer sets and the probes for mAdipoR1 and -R2 were as follows: the forward primer for mAdipoR1 was acgttgagagatcatcccgtat, the reverse primer ctctgtgtggatgccaagaat, and the probe cctgctacatggcca cagaccacct; the forward primer for mAdipoR2 was tcccaggaagatgaagggtttat, the reverse primer tccatcctgctgatagcatga, and the probe atgccccgtcctacagg ccc. For quantification of the other genes, we used a set of predesigned primers and a probe for each gene (Assay on Demand; Applied Biosystems, Foster City, CA). The relative amount of each transcript was normalized to the amount of  $\beta$ -actin transcript in the same cDNA (19).

**Isolation of adipocytes and stromal-vascular cells.** Isolation of adipocytes and stromal-vascular cells from adipose tissues were performed as described previously, with slight modification (13). Epididymal adipose tissues were isolated from mice and were minced into fine pieces. Minced tissues were incubated in Dulbecco's modified Eagle's medium supplemented with 5 mg/ml collagenase (Sigma, St. Louis, MO) and 2.5% BSA (Sigma) at 37°C for 40–45 min. Digested samples were passed through a sterile 250- $\mu$ m nylon mesh and centrifuged. The pellet cells and the floating cells were washed twice with PBS and collected as the stromal-vascular cells and adipocytes, respectively. In the experiments of direct action of PPAR agonists on primary adipocytes and stromal-vascular cells,  $1 \times 10^6$  isolated adipocytes were

seeded onto 24-well plates in Dulbecco's modified high-glucose Eagle's medium (DMEMH) supplemented with 20% charcoal-treated FCS, and  $1 \times 10^5$  isolated stromal-vascular cells were seeded onto 96-well plates in DMEMH supplemented with 20% charcoal-treated FCS and mouse granulocyte colony-stimulating factor (R&D Systems). Then, the cells were incubated with 3  $\mu$ mol/l Wy-14,643 or 0.3  $\mu$ mol/l rosiglitazone for 24 h and then harvested to isolate total RNA.

**Studies with 3T3-L1 adipocytes and peritoneal macrophages.** Mouse 3T3-L1 cells were grown in DMEMH supplemented with 10% FCS. Induction of adipogenic differentiation was carried out according to a method described previously (8). By day 8, 3T3-L1 adipocytes were incubated with 30  $\mu$ mol/l Wy-14,643 or 0.3  $\mu$ mol/l rosiglitazone in DMEMH supplemented with 1% BSA for 18 h, and then 1 ng/ml TNF- $\alpha$  was added. The cells were harvested 6 h after TNF- $\alpha$  addition to isolate total RNA. Peritoneal macrophages were isolated as previously described (26). In brief, macrophages were isolated 4 days after intraperitoneal injection of 3 ml thioglycolate medium (Sigma) to male C57BL/6j mice. Then, 1,000,000 cells were plated onto 24-well plates in RPMI-1640/10% FBS (vol/vol) and incubated for 4 h. Then, the macrophages were incubated with 30  $\mu$ mol/l Wy-14,643, 100  $\mu$ mol/l fenofibrate, or 0.3  $\mu$ mol/l rosiglitazone in the presence of 0.1  $\mu$ g/ml lipopolysaccharide for 24 h and then harvested to isolate total RNA. The concentrations of the compounds in the in vitro cell culture experiments were chosen according to previous studies, and they were comparable to those to which PPAR $\alpha$  in animal tissues were exposed in previous in vivo studies (8,10–12,27,28).

**Statistical analysis.** Data are the means  $\pm$  SE. Student's *t* test was used for statistical comparison.  $P < 0.05$  was considered statistically significant.

## RESULTS

**A PPAR $\alpha$  agonist improves insulin resistance in KKAY mice, and a combination of a PPAR $\alpha$  agonist and a PPAR $\gamma$  agonist enhances the antidiabetic effects of the PPAR $\gamma$  agonist.** To examine the antidiabetic effects of a dual activation of PPAR $\alpha$  and  $\gamma$  in comparison with a single activation of each alone, we treated obese diabetic KKAY mice with a PPAR $\gamma$  agonist, rosiglitazone, a PPAR $\alpha$  agonist, Wy-14,643, or a combination of the two for 8 weeks. We then examined the glucose and lipid metabolism and insulin sensitivity of these mice. Because it has been previously reported that PPAR $\alpha$  agonists reduce food intake in rodents (28), pair-fed control mice were given daily the same amount of food as that consumed by Wy-14,643-treated mice (Fig. 1F). As shown in Fig. 1, Wy-14,643 treatment significantly ameliorated hyperglycemia (Fig. 1A), hyperinsulinemia (Fig. 1B), and hyperlipidemia (Fig. 1C and D) compared with ad libitum-fed vehicle-treated KKAY mice. Because these effects of Wy-14,643 were significant even in comparison with pair-fed mice (Fig. 1A–D), it is likely that Wy-14,643 actually exerted its antidiabetic effects via a mechanism other than decreased food intake. Blood glucose (Fig. 1A) and plasma insulin levels (Fig. 1B) in the rosiglitazone-treated mice were significantly lower than those in the ad libitum-fed vehicle-treated mice. However, the serum lipid levels of the rosiglitazone-treated mice were significantly higher than those of the Wy-14,643-treated mice (Fig. 1C and D). Interestingly, a combination of rosiglitazone and Wy-14,643 completely normalized the hyperglycemia (Fig. 1A), hyperinsulinemia (Fig. 1B), and hyperlipidemia (Fig. 1C and D) observed in KKAY mice, and, in particular, the blood glucose (Fig. 1A) and lipid levels (Fig. 1C and D) in the combined rosiglitazone- and Wy-14,643-treated mice were significantly lower than those in the rosiglitazone-treated mice. Furthermore, serum lipid levels in the combined rosiglitazone- and Wy-14,643-treated mice were significantly lower than those in the Wy-14,643-treated mice (Fig. 1C and D). Although body weight was slightly lower in the Wy-14,643-treated mice ( $P = 0.093$ ) than in the pair-fed vehicle-treated mice, the difference was not statistically significant (Fig. 1E).

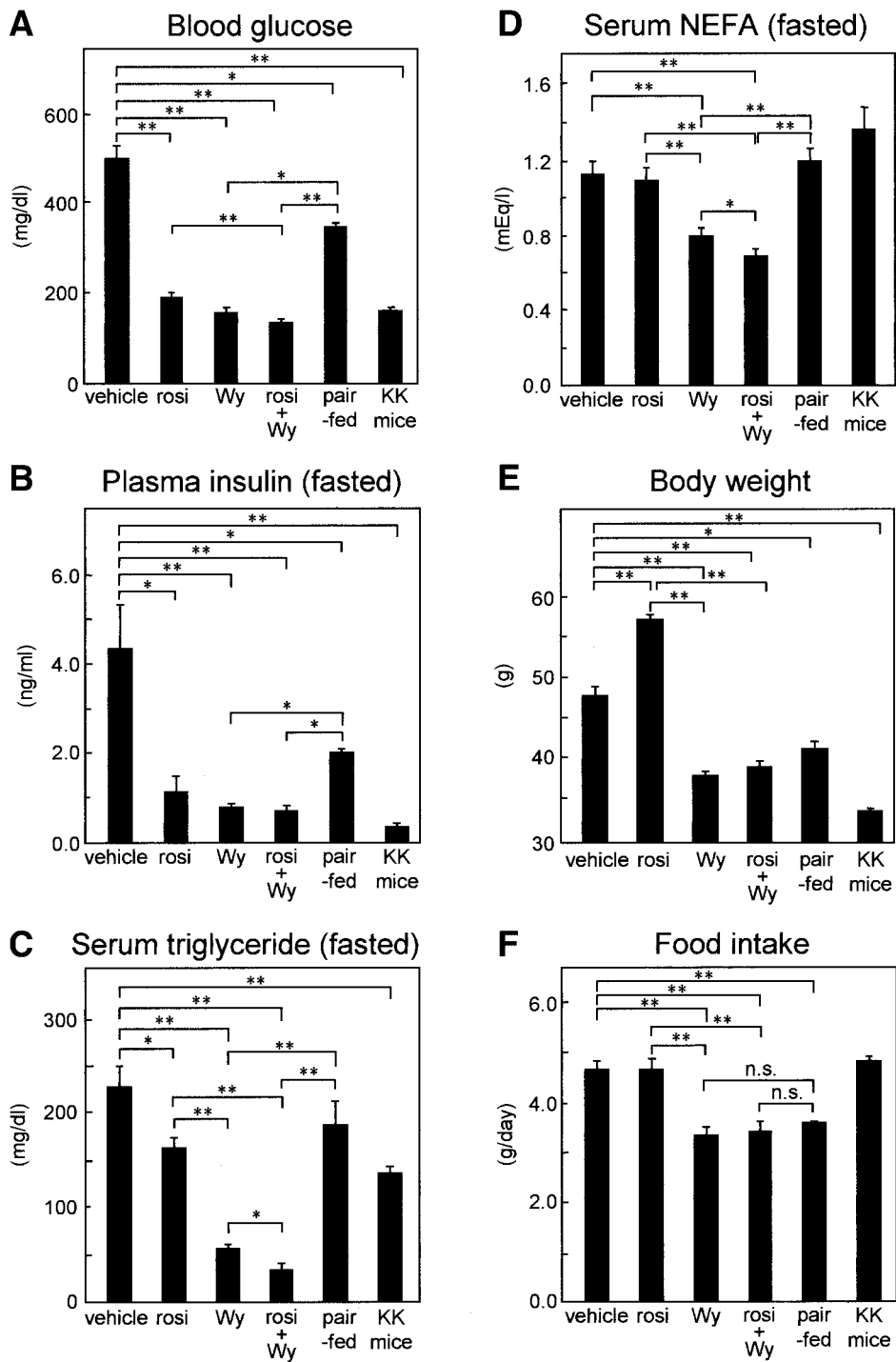


FIG. 1. Effects of rosiglitazone, Wy-14,643, or both rosiglitazone and Wy-14,643 treatment for 8 weeks on serum parameters and body weight in KKAY mice. Panels show blood glucose (A), fasting plasma insulin (B), fasting serum triglycerides (C), fasting NEFA (D), body weight (E), and food intake (F) of male KKAY mice treated with 0.01% rosiglitazone (rosi), 0.05% Wy-14,643 (Wy), or both 0.01% rosiglitazone and 0.05% Wy-14,643 (rosi+Wy) as food admixture for 8 weeks while on the high-fat diet. The same amount of food was given to the pair-fed group as to mice treated with Wy-14,643. Age-matched wild-type KK mice given normal chow were used as normal controls. Fasting parameters were measured after a 24-h fast. Each bar represents the means  $\pm$  SE ( $n = 4$  for pair-fed group,  $n = 6$  for other groups). \* $P < 0.05$ ; \*\* $P < 0.01$ . NEFA, nonesterified fatty acid; n.s., not significant.

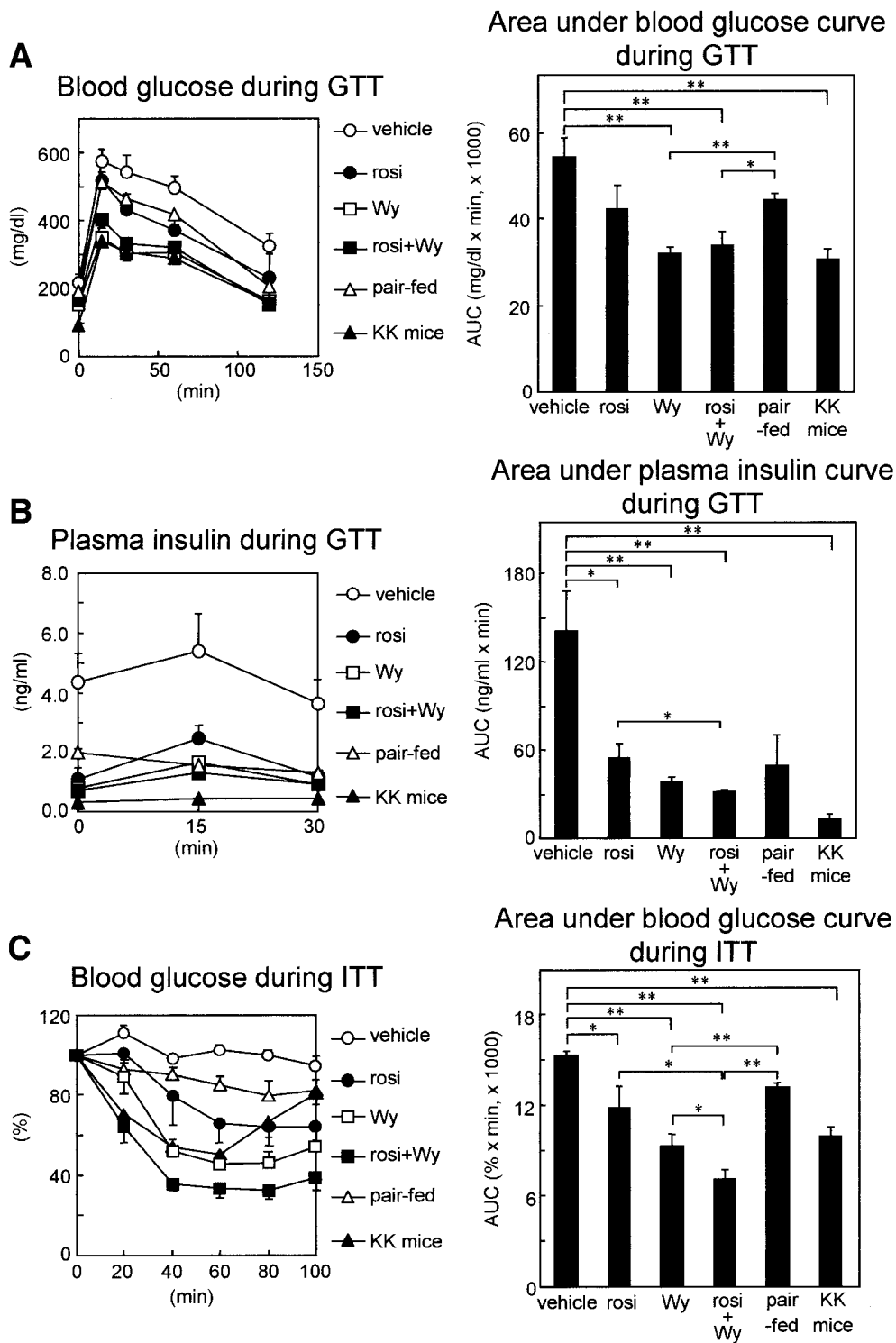
We next examined the effects of PPAR $\alpha$  and  $\gamma$  activation on the improvement of insulin resistance in more detail, using a glucose tolerance test and insulin tolerance test. Blood glucose levels during both tests in Wy-14,643-treated mice were significantly lower than those in pair-fed vehicle-treated mice (Fig. 2A and C), suggesting that Wy-14,643 treatment ameliorated insulin resistance. Again, the combination of rosiglitazone and Wy-14,643 ameliorated insulin resistance more effectively than rosiglitazone or Wy-14,643 alone (Fig. 2B and C).

**Adipocyte hypertrophy is prevented by a PPAR $\alpha$  agonist.** It has been reported that PPAR $\alpha$  agonists prevent high-fat diet-induced obesity (10). Thus, we measured

epididymal and subcutaneous WAT and intrascapular brown adipose tissue (BAT) weights. Wy-14,643 decreased both WAT and BAT weights compared with pair-fed mice (Fig. 3A–C), suggesting that PPAR $\alpha$  activation prevents obesity. The combination of rosiglitazone and Wy-14,643 decreased only epididymal WAT weights compared with pair-fed mice (Fig. 3A). In contrast, rosiglitazone increased subcutaneous WAT and BAT weights compared with vehicle (Fig. 3B and C).

We have previously shown that PPAR $\gamma$  activation by its agonist or a moderate reduction of PPAR $\gamma$  activity prevents adipocyte hypertrophy, which results in an improvement in insulin resistance (7). We next attempted to clarify

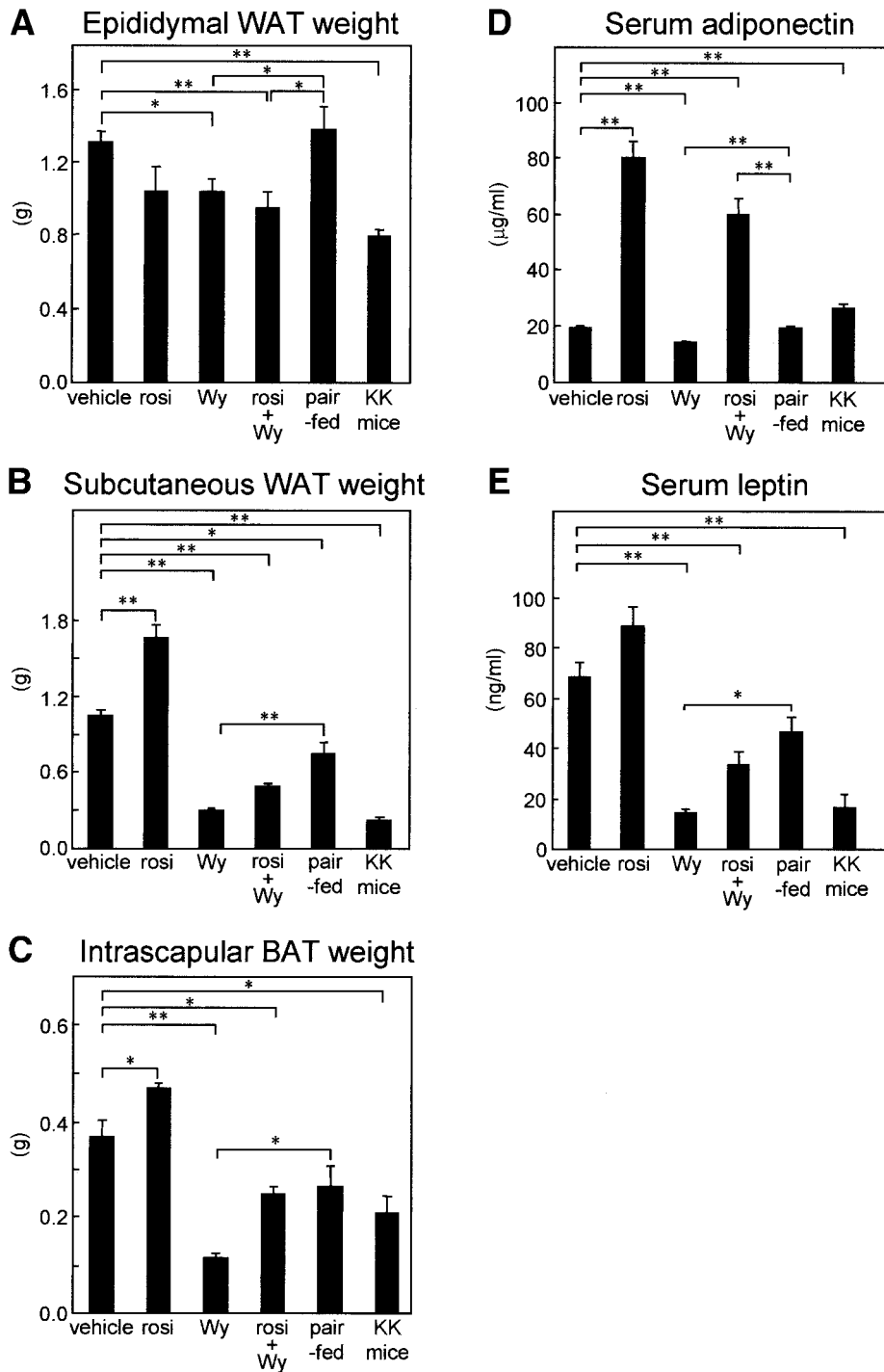




**FIG. 2.** Effects of rosiglitazone, Wy-14,643, or both rosiglitazone and Wy-14,643 treatment for 8 weeks on glucose tolerance and insulin sensitivity in KKAY mice. We measured blood glucose (A) and plasma insulin (B) during oral glucose tolerance tests (GTTs) and blood glucose during insulin tolerance tests (ITTs) (C) of male KKAY mice treated with 0.01% rosiglitazone (rosi), 0.05% Wy-14,643 (Wy), or both 0.01% rosiglitazone and 0.05% Wy-14,643 (rosi+Wy) as a food admixture for 8 weeks while on the high-fat diet. The same amount of food was given to the pair-fed group as to mice treated with Wy-14,643. Age-matched wild-type KK mice given normal chow were used as normal controls. Oral glucose tolerance tests were performed by oral gavage of 0.75 g/kg body wt glucose after 24 h fasting followed by blood sampling at the indicated time. Insulin tolerance tests were performed by 1.5 units/kg body wt i.p. insulin followed by blood sampling at the indicated time. Each bar represents the means  $\pm$  SE ( $n = 4$  for pair-fed group,  $n = 6$  for other groups).  $\circ$ , vehicle;  $\bullet$ , rosiglitazone;  $\square$ , Wy-14,643;  $\blacksquare$ , both rosiglitazone and Wy-14,643;  $\triangle$ , pair-fed;  $\blacktriangle$ , KK mice. \* $P < 0.05$ ; \*\* $P < 0.01$ .

whether adipocyte hypertrophy and increased fat pad weight are suppressed by PPAR $\alpha$  activation. The size of the adipose cells in epididymal WAT was increased in KKAY mice (Fig. 4A, upper left) compared with the wild-type control KK mice (Fig. 4A, lower right). Very interestingly, the size of the adipose cells in epididymal WAT from Wy-14,643-treated mice (Fig. 4A, upper right) was dramatically decreased compared with that from pair-fed mice (Fig. 4A, lower middle) and was comparable to that in the wild-type control KK mice (Fig. 4A, lower right). Although rosiglitazone treatment (Fig. 4A, upper middle) increased

the ratio of small adipocytes in epididymal WAT compared with vehicle treatment (Fig. 4A, upper left), the changes were moderate compared with Wy-14,643 treatment (Fig. 4A, upper right). The size of the adipose cells in epididymal WAT from mice treated with a combination of rosiglitazone and Wy-14,643 (Fig. 4A, lower left) was also smaller than that from pair-fed mice (Fig. 4A, lower middle). The size of the adipose cells in subcutaneous WAT from Wy-14,643-treated mice was also decreased compared with that from pair-fed mice (Fig. 4B). There were small nucleated cells and macrophage-specific antigen F4/80-

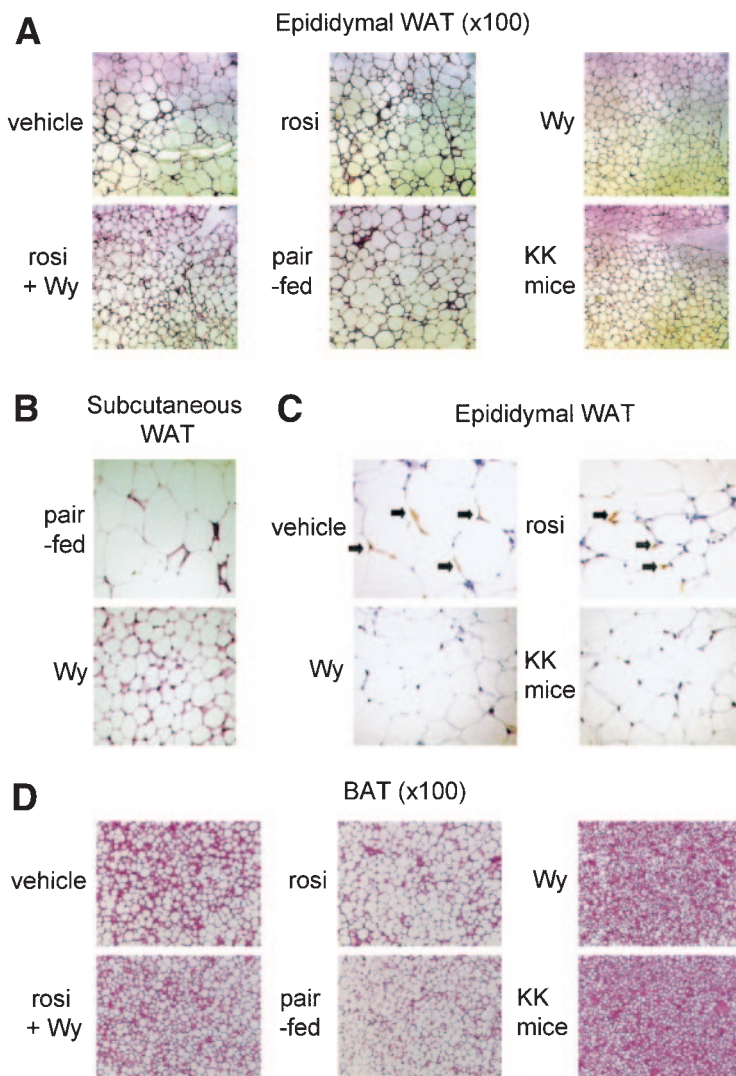


**FIG. 3.** Effects of rosiglitazone, Wy-14,643, or both rosiglitazone and Wy-14,643 treatment for 8 weeks on fat pad weight and serum adipokines in KKAY mice. Panels show epididymal WAT weight (A), subcutaneous WAT weight (B), intrascapular BAT weight (C), serum adiponectin levels (D), and serum leptin levels (E) of male KKAY mice treated with 0.01% rosiglitazone (rosi), 0.05% Wy-14,643 (Wy), or both 0.01% rosiglitazone and 0.05% Wy-14,643 (rosi+Wy) as a food admixture for 8 weeks while on the high-fat diet. The same amount of food was given to the pair-fed group as to mice treated with Wy-14,643. Age-matched wild-type KK mice given normal chow were used as normal controls. Each bar represents the means  $\pm$  SE ( $n = 4$  for pair-fed group,  $n = 6$  for other groups). \* $P < 0.05$ ; \*\* $P < 0.01$ .

expressing cells in the interstitial spaces between adipocytes in WAT of vehicle-treated KKAY mice (Fig. 4C). In contrast, there were almost no such cells in the WAT of Wy-14,643-treated mice (Fig. 4C), suggesting that macrophage infiltration to WAT may be suppressed by Wy-14,643 treatment, whereas the effect of rosiglitazone treatment seemed to be faint. We obtained similar results with BAT (Fig. 4D), except that the size of the adipocytes in BAT from rosiglitazone-treated mice (Fig. 4D, upper middle left).

We next studied whether the levels of expression of molecules secreted from WAT that regulate insulin sensi-

tivity were changed by PPAR $\alpha$  activation. As reported previously (9), serum adiponectin levels in rosiglitazone-treated mice were higher by fourfold than those in vehicle-treated mice (Fig. 3D). In contrast, serum adiponectin levels in Wy-14,643-treated mice were slightly lower than those in pair-fed mice, suggesting that the improvement in insulin resistance by Wy-14,643 was not caused by increased gene expression or secretion of adiponectin (Fig. 3D). The combination of rosiglitazone and Wy-14,643 increased serum adiponectin levels approximately threefold above the vehicle (Fig. 3D). Serum leptin levels in KKAY mice were increased by overexpression of agouti compared with those in wild-type KK mice (Fig. 3E). Wy-14,643



**FIG. 4.** Histological analyses of WAT and BAT from KKAY mice treated with rosiglitazone, Wy-14,643, or both rosiglitazone and Wy-14,643 for 8 weeks. Panels show the sections of epididymal WAT (A and C), subcutaneous WAT (B), and BAT (D), which were stained with hematoxylin and eosin (A, B, and D) or anti-F4/80 antibody (C), of male KKAY mice were treated with 0.01% rosiglitazone (rosi), 0.05% Wy-14,643 (Wy), or both 0.01% rosiglitazone and 0.05% Wy-14,643 (rosi+Wy) as a food admixture for 8 weeks while on the high-fat diet. The same amount of food was given to the pair-fed group as to mice treated with Wy-14,643. Age-matched wild-type KK mice given normal chow were used as normal controls. Arrows indicate F4/80-expressing cells.

treatment decreased the serum leptin levels to levels comparable to wild-type KK mice (Fig. 3E). Although rosiglitazone treatment slightly ( $P = 0.057$ ) increased serum leptin levels, combined rosiglitazone and Wy-14,643 treatment decreased them compared with vehicle (Fig. 3E).

**A PPAR $\alpha$  agonist increases molecules involved in fatty acid combustion in liver and BAT.** PPAR $\alpha$  is abundantly expressed in liver and BAT in rodents, and PPAR $\alpha$  activation induces fatty acid combustion (29,30). Thus, we examined the gene expression of molecules involved in fatty acid oxidation and lipolysis, using quantitative PCR analyses. As shown in Fig. 5B and C, the expression of uncoupling protein (UCP)-1 and  $\beta$ 3-adrenergic receptor in BAT were decreased in vehicle-treated KKAY mice compared with wild-type KK mice, consistent with the adipocyte hypertrophy observed in KKAY mice (Fig. 5C). Wy-14,643 treatment increased the expression of UCP2 in liver (Fig. 5A) and the expression of UCP1 and  $\beta$ 3-adrenergic receptor in BAT (Fig. 5B and C). In contrast, rosiglitazone treatment decreased the expression of UCP1 and  $\beta$ 3-adrenergic receptor in BAT compared with vehicle (Fig. 5B and C). Combined treatment with rosiglitazone and Wy-14,643 increased the expression of UCP2 (Fig. 5A), but it did not affect the expression of UCP1 and  $\beta$ 3-adrenergic receptor in BAT compared with vehicle (Fig. 5B and C). Taken together, these findings suggest that the

induction of molecules involved in fatty acid combustion in liver and BAT is, at least in part, responsible for the prevention of adipocyte hypertrophy by PPAR $\alpha$  activation. **A PPAR $\alpha$  agonist suppresses inflammation in WAT.** Recently, it was proposed that obesity-related insulin resistance may be a chronic inflammatory disease initiated in adipose tissue (13,14). Although it has been reported that rosiglitazone suppressed the expression of inflammation genes in WAT of obese diabetic *ob/ob* mice (14), the effects of PPAR $\alpha$  agonists on inflammation in WAT have not yet been studied in detail. We studied whether Wy-14,643 suppressed the expression of inflammatory genes in WAT. As shown in Fig. 5D–F, the expression of TNF- $\alpha$ , monocyte chemoattractant protein (MCP)-1, and macrophage antigen-1 in WAT of vehicle-treated KKAY mice were significantly increased compared with wild-type KK mice, suggesting that the accumulation of macrophages and inflammatory responses were induced in WAT of KKAY mice. Interestingly, Wy-14,643 treatment suppressed the increased expression of TNF- $\alpha$ , MCP-1, and macrophage antigen-1 in WAT compared with vehicle (Fig. 5D–F), which was consistent with the suppression of macrophage infiltration into WAT by Wy-14,643 treatment (Fig. 4C). Rosiglitazone treatment partially suppressed the expression of MCP-1 in WAT (Fig. 5F), but it did not affect the expression of TNF- $\alpha$  and macrophage antigen-1 compared

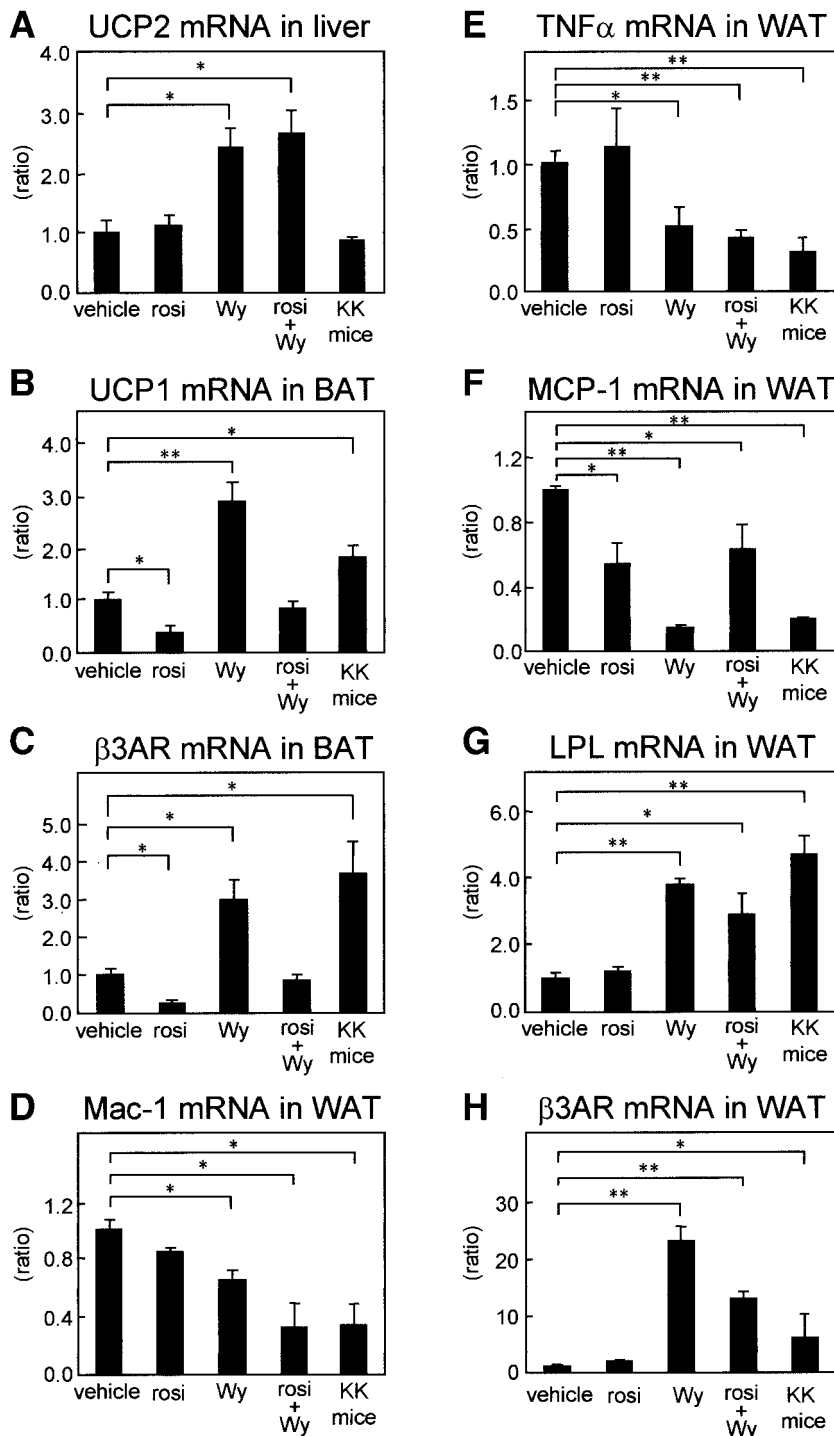


FIG. 5. Effects of rosiglitazone, Wy-14,643, or both rosiglitazone and Wy-14,643 treatment for 8 weeks on expression of molecules involving fatty acid combustion and inflammation in liver, BAT, and WAT from KKAY mice. Panels show amounts of mRNAs of UCP2 in liver (A); UCP1 (B) and  $\beta$ 3-adrenergic receptor (C) in BAT; and macrophage antigen (Mac)-1 (D), TNF- $\alpha$  (E), MCP-1 (F), lipoprotein lipase (G), and  $\beta$ 3-adrenergic receptor ( $\beta$ 3AR) (H) in epididymal WAT of male KKAY mice treated with 0.01% rosiglitazone (rosi), 0.05% Wy-14,643 (Wy), or both 0.01% rosiglitazone and 0.05% Wy-14,643 (rosi+Wy) as food admixture for 8 weeks while on the high-fat diet. Age-matched wild-type KK mice given normal chow were used as normal controls. Amounts of the mRNAs of molecules indicated above were quantified by a real-time PCR method as described in RESEARCH DESIGN AND METHODS. The results are expressed as the ratio of the value of vehicle-treated KKAY mice. Each bar represents the means  $\pm$  SE ( $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ .

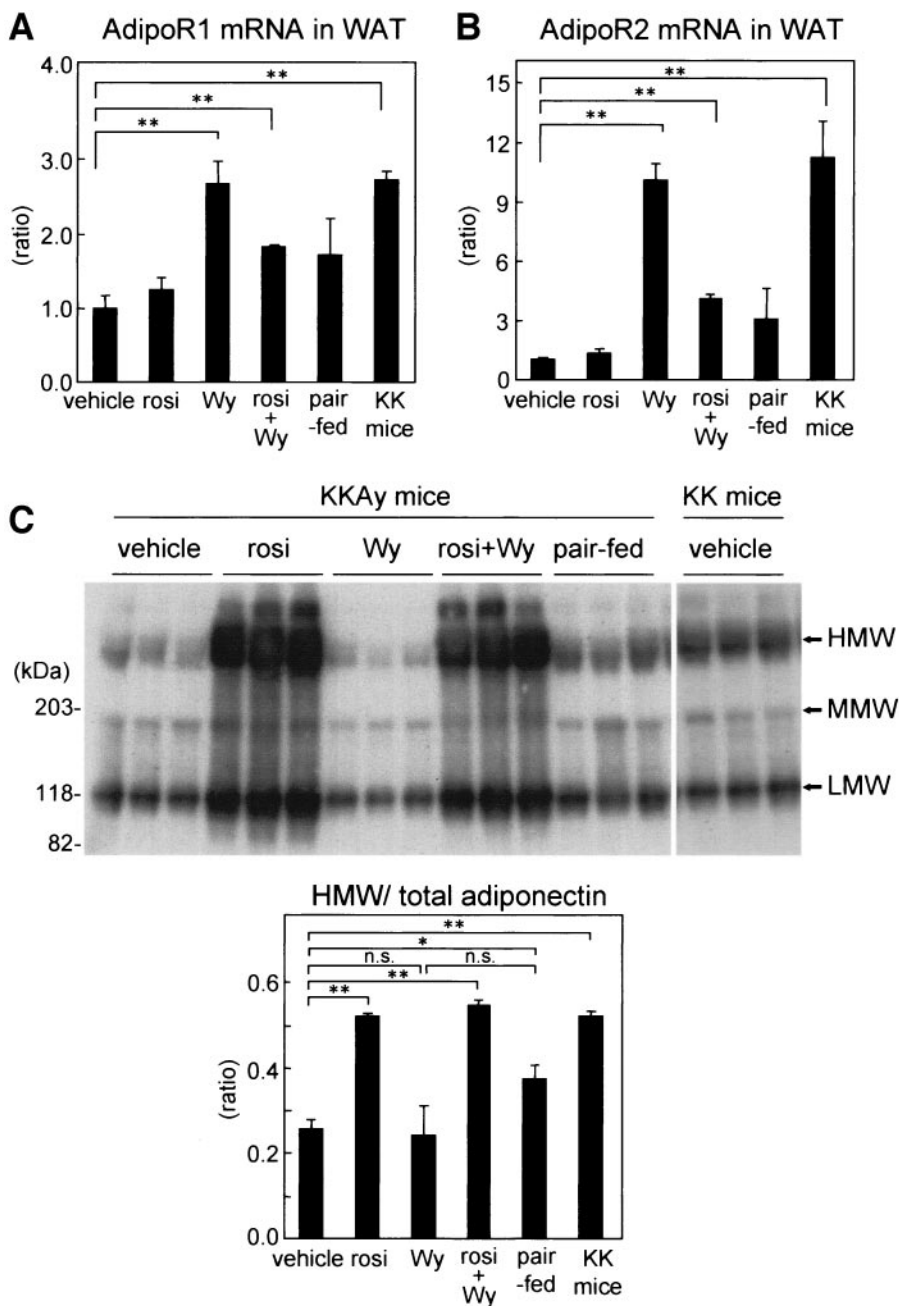
with vehicle (Fig. 5D and E). Combined rosiglitazone and Wy-14,643 treatment, as well as Wy-14,643 treatment, suppressed the expression of these genes compared with vehicle (Fig. 5D–F).

It has been reported that MCP-1 decreased the expression of genes involved in adipocyte function and attenuated insulin sensitivity in cultured adipocytes (31). Thus, we examined the effects of PPAR $\alpha$  activation on the expression of lipoprotein lipase and  $\beta$ 3-adrenergic receptor in WAT. The expression of lipoprotein lipase and  $\beta$ 3-adrenergic receptor were decreased in WAT of vehicle-treated KKAY mice compared with wild-type KK mice (Fig. 5G and H). Wy-14,643 treatment increased the expression of li-

poprotein lipase and  $\beta$ 3-adrenergic receptor in WAT, whereas rosiglitazone treatment showed no effect (Fig. 5G and H). Taken together, these data suggest that Wy-14,643 suppressed inflammation in WAT and normalized gene expression, which were dysregulated by obesity-associated adipocyte hypertrophy and inflammation.

**A PPAR $\alpha$  agonist increases the expression of adiponectin receptors (AdipoRs) in WAT, whereas a PPAR $\gamma$  agonist increases the ratio of high molecular weight multimers of adiponectin to total adiponectin.** We previously reported that AdipoR1 and -2 are downregulated in WAT, BAT, and skeletal muscles in obese diabetic *ob/ob* mice, which are correlated with decreased adiponec-





**FIG. 6.** Effects of rosiglitazone, Wy-14,643, or both rosiglitazone and Wy-14,643 treatment for 8 weeks on expression of AdipoR1/R2 in WAT and multimer forms of serum adiponectin in KKAy mice. Panels show amounts of mRNAs of AdipoR1 (A) and AdipoR2 (B) in epididymal WAT of male KKAy mice treated with 0.01% rosiglitazone (rosi), 0.05% Wy-14,643 (Wy), or both 0.01% rosiglitazone and 0.05% Wy-14,643 (rosi+Wy) as food admixture for 8 weeks while on the high-fat diet. The same amount of food was given to the pair-fed group as to mice treated with Wy-14,643. Age-matched wild-type KK mice given normal chow were used as normal controls. Amounts of the mRNAs of AdipoR1/R2 were quantified by a real-time PCR method as described in RESEARCH DESIGN AND METHODS. The relative amount of each transcript was normalized to the amount of  $\beta$ -actin transcript in the same cDNA. The results are expressed as the ratio of the value of vehicle-treated KKAy mice. Serum of KKAy mice treated with compounds mentioned above was subjected to SDS-PAGE under nonreducing, non-heat-denaturing conditions, and multimer forms of adiponectin were detected, using anti-adiponectin antibody (C). D: The quantitative results of upper panels. Each bar represents the means  $\pm$  SE ( $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ . LMW, low molecular weight; n.s., not significant.

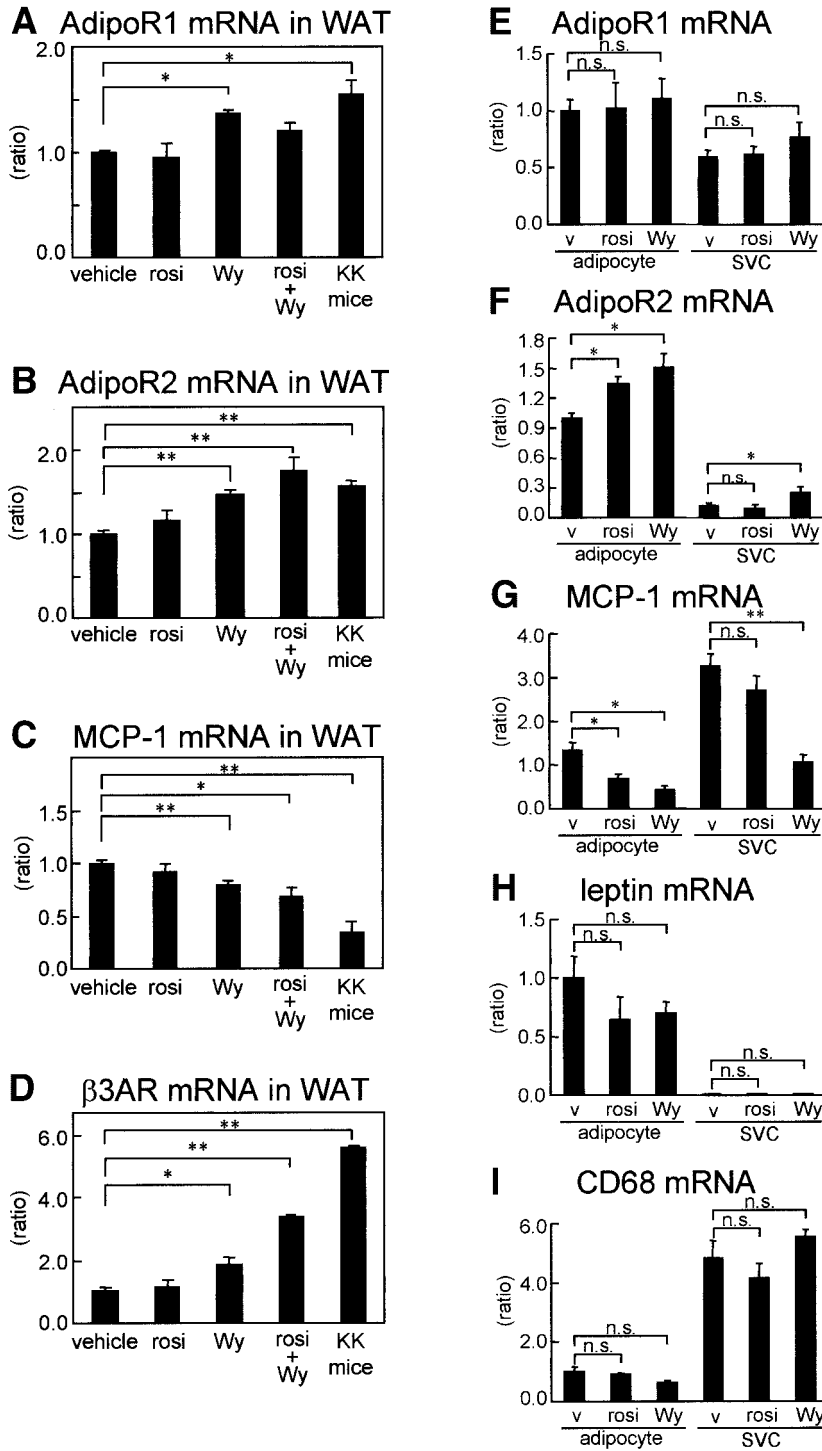
tin sensitivity (32). As shown in Fig. 6A and B, the expression of AdipoR1 and -2 was decreased in WAT of vehicle-treated KKAy mice compared with wild-type KK mice. Wy-14,643 treatment almost completely reversed the decrease in AdipoR1 and -2 expression in KKAy mice (Fig. 6A and B). In contrast, the expression levels of AdipoR1 and -2 were not affected by rosiglitazone treatment, whereas combined rosiglitazone and Wy-14,643 treatment partially reversed the decrease in these genes (Fig. 6A and B). We also obtained similar results in BAT (data not shown).

Adiponectin is known to form characteristic multimers (33,34). It was recently reported that the increase in the ratio of the high molecular weight (HMW) form to total adiponectin is correlated with an improvement in insulin sensitivity by TZD treatment (35). Here, we studied whether a PPAR $\alpha$  agonist affects the forms of serum adiponectin. As shown in Fig. 6C, the ratio of HMW to total adiponectin was decreased in vehicle-treated KKAy mice

compared with wild-type KK mice. The restriction of food intake by pair-fed mice partially restored the decrease in the ratio of HMW to total adiponectin in KKAy mice (Fig. 6C). Wy-14,643 treatment did not affect the ratio of HMW to total adiponectin, whereas rosiglitazone treatment dramatically increased total adiponectin and the ratio of HMW to total adiponectin (Fig. 6C). Treatment with a combination of rosiglitazone and Wy-14,643 showed results similar to rosiglitazone treatment (Fig. 6C).

**A PPAR $\alpha$  agonist directly increased AdipoR expression and suppressed MCP-1 expression.** There is a possibility that long-term treatment with Wy-14,643 indirectly increased the gene expression in the WAT of KKAy mice via an improvement in insulin resistance or some other mechanism. Therefore, we examined the effects of short-term treatment with Wy-14,643 on the expression of AdipoR1, AdipoR2, MCP-1, and  $\beta$ 3-adrenergic receptor in WAT of KKAy mice. As shown in Fig. 7A–D, treatment with





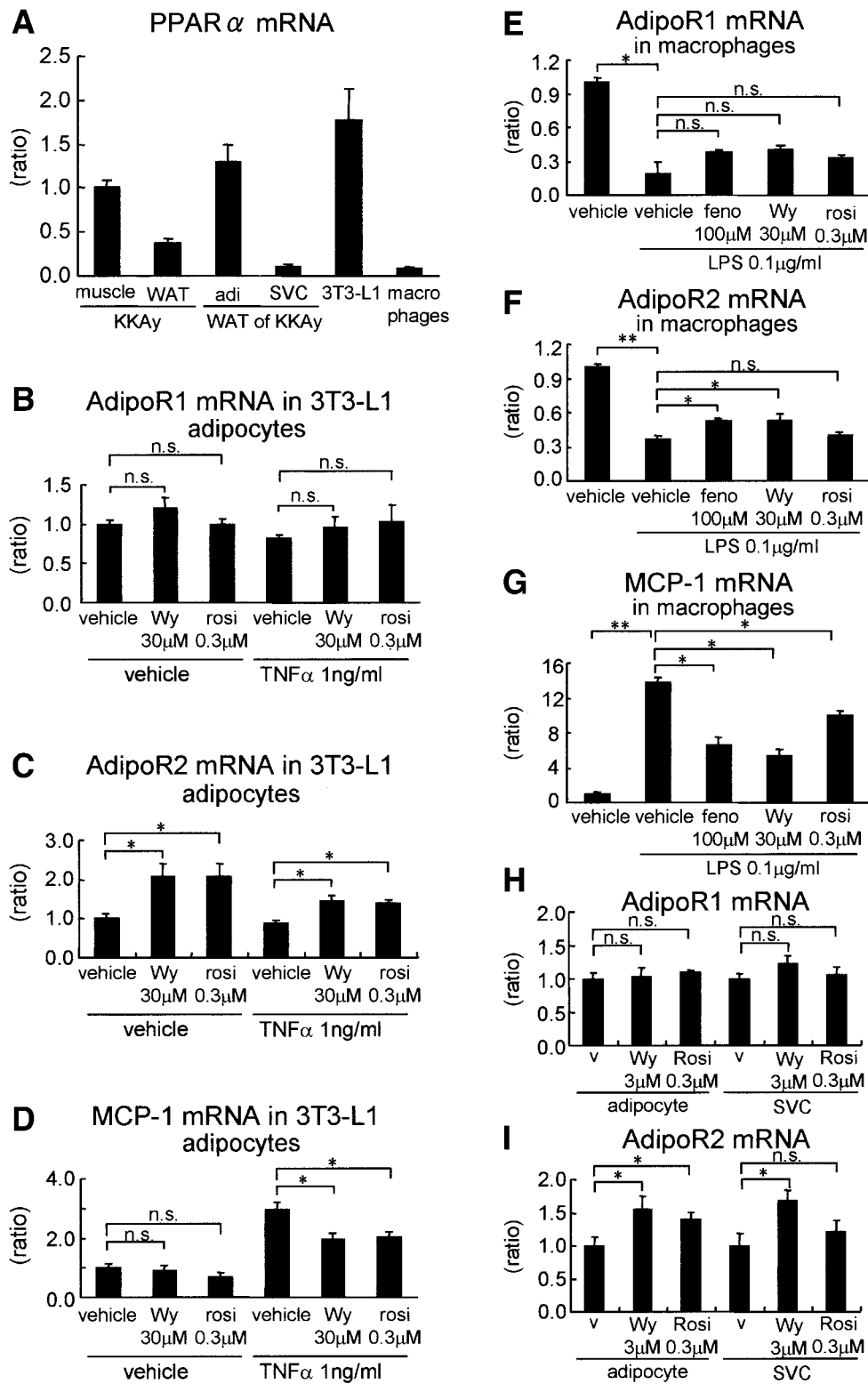
Wy-14,643 for 3 days increased the expression of AdipoR1, AdipoR2, and  $\beta$ 3-adrenergic receptor and decreased the expression of MCP-1 in WAT of KKAY mice. We obtained similar results with the combined treatment of rosiglitazone and Wy-14,643 (Fig. 7A–D).

WAT of obese diabetic mice is thought to consist of adipocytes and stromal vascular cells, such as infiltrated macrophages (13,14). We next tried to determine in which cell type the observed effects occur by analyzing the adipocyte and stromal-vascular cell subfractions of adipose tissue separately. As shown in Fig. 7E and G, treatment with Wy-14,643 for 3 days increased the expression of AdipoR2 and decreased the expression of MCP-1 in both

adipocytes and stromal-vascular cells, whereas rosiglitazone treatment showed similar effects only in adipocytes. The expression pattern of leptin and CD68, markers of adipocytes and stromal-vascular cells, respectively, confirmed that the fractionation was performed properly (Fig. 7H and I).

We next examined the expression of PPAR $\alpha$  in the adipose tissue fractions, cultured 3T3-L1 adipocytes, and peritoneal macrophages to determine whether the effects of Wy-14,643 on adipocytes and macrophages were mediated by PPAR $\alpha$ . PPAR $\alpha$  expression was observed in both the adipose fraction and 3T3-L1 adipocytes as well as in skeletal muscle of KKAY mice (Fig. 8A), whereas PPAR $\alpha$

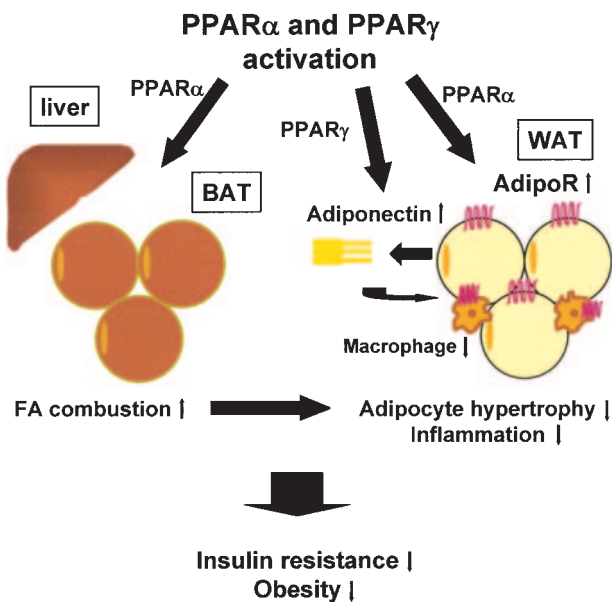
FIG. 7. Effects of rosiglitazone, Wy-14,643, or both rosiglitazone and Wy-14,643 treatment for 3 days on expression of AdipoR1/R2, MCP-1, and  $\beta$ 3-adrenergic receptor in WAT of KKAY mice and action of Wy-14,643 on expression of AdipoR1/R2 and MCP-1 in adipocyte (adi) and stromal-vascular cell subfractions of WAT. Panels show amounts of mRNAs of AdipoR1 (A), AdipoR2 (B), MCP-1 (C), and  $\beta$ 3-adrenergic receptor ( $\beta$ 3AR) (D) in epididymal WAT of male KKAY mice treated with 0.01% rosiglitazone (rosi), 0.05% Wy-14,643 (Wy), or both 0.01% rosiglitazone and 0.05% Wy-14,643 (rosi+Wy) as a food admixture for 3 days while on the high-fat diet. Age-matched wild-type KK mice given normal chow were used as normal controls. Amounts of mRNAs of AdipoR1 (E), AdipoR2 (F), and MCP-1 (G) from adipocyte and stromal-vascular cell subfractions of WAT of KKAY mice treated with 0.01% rosiglitazone or 0.05% Wy-14,643 for 3 days while on the high-fat diet. Amounts of the mRNAs of molecules indicated above were quantified by a real-time PCR method as described in RESEARCH DESIGN AND METHODS. The relative amount of each transcript was normalized to the amount of  $\beta$ -actin transcript in the same cDNA. The results are expressed as the ratio of the value of vehicle (v). Each bar represents the means  $\pm$  SE ( $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ . n.s., not significant; SVC, stromal-vascular cell.



**FIG. 8.** Direct action of Wy-14,643 on expression of AdipoR1/R2 and MCP-1 in cultured cells. Panels show amounts of PPAR $\alpha$  mRNAs in tissues (skeletal muscle and WAT of KKAy mice), subfractions of WAT (adipocytes and stromal-vascular cells), and cultured cells (3T3-L1 adipocytes and isolated mice peritoneal macrophages) (A). Also shown are amounts of mRNAs of AdipoR1 (B), AdipoR2 (C), and MCP-1 (D) from 3T3-L1 adipocytes incubated with vehicle (v), 30  $\mu$ mol/l Wy-14,643 (Wy), or 0.3  $\mu$ mol/l rosiglitazone (rosi) for 18 h followed by stimulation with or without 1 ng/ml TNF- $\alpha$  for 6 h. We determined the amounts of mRNA of AdipoR1 (E), AdipoR2 (F), and MCP-1 (G) in peritoneal macrophages incubated with vehicle or with 30  $\mu$ mol/l Wy-14,643 (Wy), 100  $\mu$ mol/l fenofibrate (feno), or 0.3  $\mu$ mol/l rosiglitazone (rosi) with 0.1  $\mu$ g/ml lipopolysaccharide (LPS). We determined the amounts of mRNA of AdipoR1 (H) and AdipoR2 (I) in primary adipocytes and stromal-vascular cells (SVC) incubated with vehicle (v), 3  $\mu$ mol/l Wy-14,643, or 0.3  $\mu$ mol/l rosiglitazone. Amounts of the mRNAs of molecules indicated above were quantified by a real-time PCR method as described in the RESEARCH DESIGN AND METHODS. The relative amount of each transcript was normalized to the amount of  $\beta$ -actin transcript in the same cDNA. The results are the ratio of the value of skeletal muscle (A) or vehicle (B-G). Each bar represents the means  $\pm$  SE ( $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ . n.s., not significant.

expression levels in stromal-vascular cells and peritoneal macrophages were much lower than those in skeletal muscle of KKAy mice (Fig. 8A). We further examined the direct action of Wy-14,643 on gene expression in cultured adipocytes or macrophages. A 24-h treatment with 30  $\mu$ mol/l Wy-14,643 significantly increased AdipoR2 expression in 3T3-L1 adipocytes (Fig. 8C), whereas AdipoR1 expression was not significantly increased (Fig. 8B). Wy-14,643 treatment suppressed the expression of MCP-1,

which was increased by 1 ng/ml TNF- $\alpha$  stimulation in 3T3-L1 adipocytes (Fig. 8D). Fenofibrate treatment (100  $\mu$ mol/l) also increased AdipoR2 expression by 25% compared with vehicle in 3T3-L1 adipocytes (data not shown). Wy-14,643 treatment as well as fenofibrate treatment suppressed the increase in MCP-1 expression caused by 0.1  $\mu$ g/ml lipopolysaccharide treatment and slightly but significantly increased AdipoR2 expression in peritoneal macrophages (Fig. 8F and G). Rosiglitazone treatment increased



**FIG. 9.** Proposed mechanisms for improvement of insulin resistance by PPAR $\alpha$  and  $\gamma$ . Activation of PPAR $\alpha$ , at least in part, directly suppresses inflammation and adipocyte hypertrophy in WAT in addition to stimulating fatty acid (FA) combustion in liver and BAT. Dual activation of PPAR $\alpha$  and  $\gamma$  enhances the action of adiponectin by increasing AdipoR expression and the ratio of HMW to total adiponectin leading to enhancement of action of adiponectin.

AdipoR2 expression only in 3T3-L1 adipocytes (Fig. 8C) and suppressed MCP-1 expression in both adipocytes and macrophages (Fig. 8D and G), whereas the effect of Wy-14,643 treatment on macrophages was more potent than that of rosiglitazone treatment (Fig. 8G). Furthermore, we examined the direct action of the lower concentration of Wy-14,643 on gene expression in primary cultured adipocytes or stromal-vascular cells. A 24-h treatment with 3  $\mu\text{mol/l}$  Wy-14,643 significantly increased AdipoR2 expression in adipocytes and stromal-vascular cells (Fig. 8D), whereas AdipoR1 expression was not significantly increased (Fig. 8H). These data suggest that Wy-14,643 directly increased AdipoR2 expression in adipocytes and suppressed MCP-1 expression in both adipocytes and macrophages.

## DISCUSSION

Recent studies have revealed that adipose tissue plays a key role in regulating whole-body glucose metabolism (13,14,36). In this study, we attempted to clarify the mechanisms by which activation of PPAR $\alpha$  and/or PPAR $\gamma$  ameliorates insulin resistance, focusing on adipose tissue. We demonstrate here that activation of PPAR $\alpha$  prevented adipocyte hypertrophy, at least in part, through mechanisms other than decreased food intake. As shown in Fig. 9, one possible and conventional mechanism for this effect is an increase in systemic energy expenditure by the induction of molecules involved in fatty acid combustion, such as UCPs in liver and BAT (Fig. 5A–C) (27,37,38). We propose that there is also another pathway in which the activation of PPAR $\alpha$  in WAT normalizes adipocyte function associated with an increase in  $\beta$ 3-adrenergic receptor, which is expressed in WAT and BAT and has been shown to play important roles in lipolysis and thermogenesis (39,40). WAT has not been considered as a main target tissue of PPAR $\alpha$  agonist so far (29,30). PPAR $\alpha$  mRNA was, however, actually detected by real-time PCR analysis in

the adipocyte subfraction of WAT and 3T3-L1 adipocytes (although the expression levels were much lower in adipocytes compared with liver), as well as in skeletal muscle (Fig. 8A). In addition, it was recently reported that PPAR $\alpha$  agonists, such as Wy-14,643, directly enhance lipolysis in isolated adipocytes (41). Thus, it is possible that high concentrations of a PPAR $\alpha$  agonist can directly activate PPAR $\alpha$  in adipocytes. It remains to be determined which tissue mainly contributes to the improvement in insulin resistance by PPAR $\alpha$  activation. To clarify this point, it is necessary to investigate the effects of PPAR $\alpha$  agonists in PPAR $\alpha$  tissue-specific knockout mice.

It was recently reported that a PPAR $\alpha/\gamma$  agonist elevated pyruvate dehydrogenase kinase isozyme 4 (PDK4) mRNA levels in liver compared with vehicle or a PPAR $\gamma$  agonist (42). In the present study, we checked PDK4 mRNA levels in liver and epididymal adipose tissue and found that PDK4 mRNA levels in epididymal adipose tissue of Wy-14,643-treated KKAY mice were significantly higher than those of vehicle-treated KKAY mice (data not shown), indicating that PPAR $\alpha$  was actually activated in adipose tissue by Wy-14,643 treatment. However, there was no significant change in the PDK4 mRNA level in liver between vehicle- and Wy-14,643-treated KKAY mice (data not shown). Precise reasons that explain differences between the results of their study and those of our current study are not known at present. However, several factors, such as animal species (rats and mice), treatment periods (1 and 8 weeks), administration route (oral gavage and mixed chow), and sampling time after the final dose (6 and 24 h) are different between the two studies, which may cause differences in gene expression of PDK4 in liver.

Here we have shown that activation of PPAR $\alpha$  suppressed obesity-induced increases in inflammatory cytokines such as TNF- $\alpha$  and MCP-1 in WAT. To the best of our knowledge, this is the first report indicating that the activation of PPAR $\alpha$  regulates inflammatory inflammation in WAT, whereas it has been reported that TZDs also suppressed the increased expression of inflammatory genes in WAT of *ob/ob* mice (14). In the current study, the efficacy of the PPAR $\alpha$  agonist appeared to be more potent than that of the PPAR $\gamma$  agonist with respect to suppression of the increased expression of inflammatory molecules in vivo. Although PPAR $\alpha$  agonists have been shown to inhibit a nuclear factor- $\kappa$ B pathway stimulated by proinflammatory substances in smooth muscle cells (43,44), the distinct mechanism by which PPAR $\alpha$  agonists suppress the expression of inflammatory cytokines in WAT remains to be clarified. However, in this study, a PPAR $\alpha$  agonist directly suppressed the increase in MCP-1 expression by proinflammatory substances in cultured adipocytes or macrophages in vitro. Therefore, it is plausible that PPAR $\alpha$  can mediate the reduction of proinflammatory substance-induced increase in MCP-1 expression in both adipocytes and infiltrated macrophages in WAT, leading to a reduction of macrophage accumulation and a reversal of adipocyte dysfunction.

Finally, we found that activation of PPAR $\alpha$  increased AdipoR1 and -2 expression in both WAT and BAT in vivo. In contrast, activation of PPAR $\alpha$  increased only AdipoR2 in cultured cells. This result is consistent with previous studies that found PPAR $\alpha$  agonists increased only AdipoR2 expression in cultured macrophages (45). Furthermore, the expression of AdipoR1 and -2 in the liver from Wy-14,643-treated KKAY mice were not significantly changed compared with those from vehicle-treated mice



(data not shown). Therefore, cofactors that are expressed in adipocytes and macrophages and bind with PPAR $\alpha$  may be involved in the regulation of AdipoR2 expression. Activation of PPAR $\gamma$  also increased the expression of AdipoR2 in adipocytes in vitro and in vivo, but it did not affect the expression of AdipoR2 in either WAT or BAT in vivo because an increase of AdipoR2 expression in stromal-vascular cells in adipose tissues was not observed after PPAR $\gamma$  activation. The increase of AdipoR2 expression in adipocytes may be caused by the secondary effect on adipocyte differentiation by PPAR $\gamma$  activation because AdipoR2 expression was dramatically increased during adipocyte differentiation (T.Y., T.K., unpublished data). It was recently reported that adiponectin inhibits lipopolysaccharide-induced inflammatory responses in adipocytes (46). Therefore, the increase of AdipoR expression by PPAR $\alpha$  activation in adipocytes may enhance adiponectin's anti-inflammatory action in WAT. To date, however, there are no data indicating to what extent increases in AdipoR1 and -2 expression contribute to the improvement in insulin resistance by PPAR $\alpha$  activation. Further studies using AdipoR knockout mice will be required to clarify this point. We also showed that activation of PPAR $\gamma$  or food restriction increased the ratio of HMW to total adiponectin and that activation of PPAR $\alpha$  did not affect the ratio. This result indicates that an improvement in adipocyte hypertrophy or a reduction in body weight was sufficient to increase the ratio of HMW to total adiponectin. Further investigations will be important to clarify how PPAR $\gamma$  agonists increase HMW adiponectin because HMW adiponectin may be the active form of this protein with respect to its glucose-lowering effect (35) and in the activation of AMP kinase (T.Y., T.K., unpublished data).

In conclusion, as shown in Fig. 9, we have proposed novel mechanisms by which the activation of PPAR $\alpha$  and  $\gamma$  can improve obesity-induced insulin resistance. First, activation of PPAR $\alpha$  suppresses inflammation and adipose hypertrophy in WAT. Second, dual activation of PPAR $\alpha$  and  $\gamma$  enhances the action of adiponectin by increasing AdipoR expression and the ratio of HMW to total adiponectin.

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