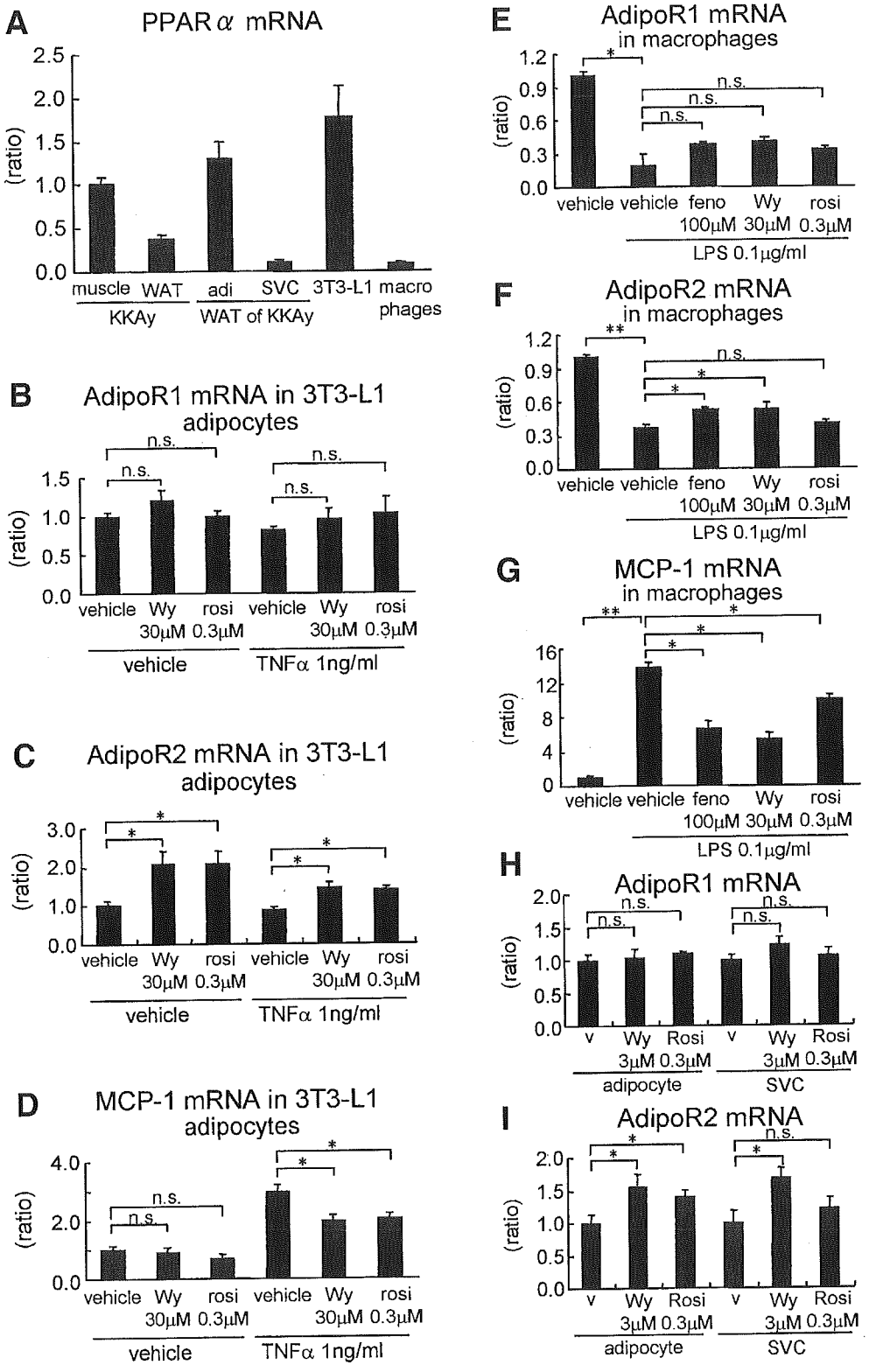

FIG. 8. Direct action of Wy-14,643 on expression of AdipoR1/R2 and MCP-1 in cultured cells. Panels show amounts of PPARα mRNAs in tissues (skeletal muscle and WAT of KKAy mice), subfractions of WAT (adipocytes and stromal-vascular cells), and cultured cells (3T3-L1 adipocytes and isolated mice peritoneal 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 μmol/l Wy-14,643 (Wy), or 0.3 μmol/l rosiglitazone (rosi) for 18 h followed by stimulation with or without 1 ng/ml TNF-α 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 μmol/l Wy-14,643 (Wy), 100 μmol/l fenofibrate (feno), or 0.3 μmol/l rosiglitazone (rosi) with 0.1 μg/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 μmol/l Wy-14,643, or 0.3 μmol/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 β-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 ± 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. 8*A*). We further examined the direct action of Wy-14,643 on gene expression in cultured adipocytes or macrophages. A 24-h treatment with 30 μmol/l Wy-14,643 significantly increased AdipoR2 expression in 3T3-L1 adipocytes (Fig. 8*C*), whereas AdipoR1 expression was not significantly increased (Fig. 8*B*). Wy-14,643 treatment suppressed the expression of MCP-1, which was increased by 1 ng/ml TNF-α stimulation in 3T3-L1 adipocytes (Fig. 8*D*). Fenofibrate treatment (100 μmol/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 μg/ml lipopolysaccharide treatment and slightly but significantly increased AdipoR2 expression in peritoneal macrophages (Fig. 8*F* and *G*). Rosiglitazone treatment increased

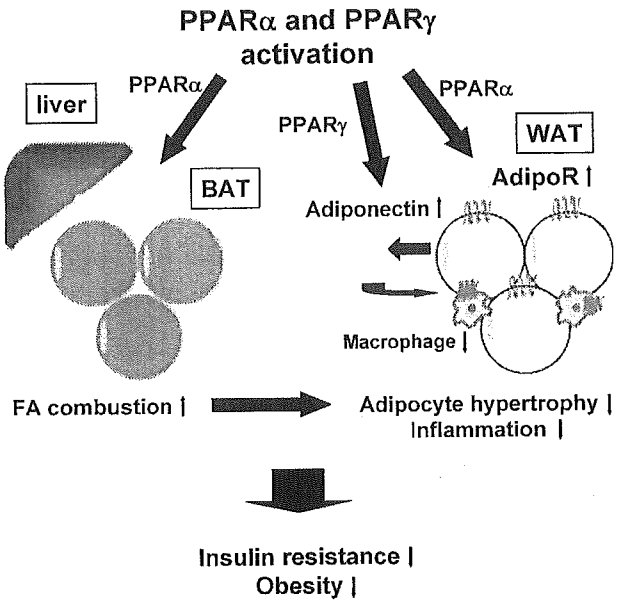

FIG. 9. Proposed mechanisms for improvement of insulin resistance by PPARα and -γ. Activation of PPARα, at least in part, directly suppresses inflammation and adipocyte hypertrophy in WAT in addition to stimulating fatty acid (FA) combustion in liver and BAT. Dual activation of PPARα and -γ enhances 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. 8*C*) and suppressed MCP-1 expression in both adipocytes and macrophages (Fig. 8*D* and *G*), whereas the effect of Wy-14,643 treatment on macrophages was more potent than that of rosiglitazone treatment (Fig. 8*G*). 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 μmol/l Wy-14,643 significantly increased AdipoR2 expression in adipocytes and stromal-vascular cells (Fig. 8*I*), whereas AdipoR1 expression was not significantly increased (Fig. 8*H*). 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 PPARα and/or PPARγ ameliorates insulin resistance, focusing on adipose tissue. We demonstrate here that activation of PPARα 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. 5*A*–*C*) (27,37,38). We propose that there is also another pathway in which the activation of PPARα in WAT normalizes adipocyte function associated with an increase in β3-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 PPARα agonist so far (29,30). PPARα 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. 8*A*). In addition, it was recently reported that PPARα agonists, such as Wy-14,643, directly enhance lipolysis in isolated adipocytes (41). Thus, it is possible that high concentrations of a PPARα agonist can directly activate PPARα in adipocytes. It remains to be determined which tissue mainly contributes to the improvement in insulin resistance by PPARα activation. To clarify this point, it is necessary to investigate the effects of PPARα agonists in PPARα tissue–specific knockout mice.

It was recently reported that a PPARα/γ agonist elevated pyruvate dehydrogenase kinase isozyme 4 (PDK4) mRNA levels in liver compared with vehicle or a PPARγ 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 PPARα 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 PPARα suppressed obesity-induced increases in inflammatory cytokines such as TNF-α and MCP-1 in WAT. To the best of our knowledge, this is the first report indicating that the activation of PPARα regulates 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 PPARα agonist appeared to be more potent than that of the PPARγ agonist with respect to suppression of the increased expression of inflammatory molecules in vivo. Although PPARα agonists have been shown to inhibit a nuclear factor-κB pathway stimulated by proinflammatory substances in smooth muscle cells (43,44), the distinct mechanism by which PPARα agonists suppress the expression of inflammatory cytokines in WAT remains to be clarified. However, in this study, a PPARα agonist directly suppressed the increase in MCP-1 expression by proinflammatory substances in cultured adipocytes or macrophages in vitro. Therefore, it is plausible that PPARα 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 PPARα increased AdipoR1 and -2 expression in both WAT and BAT in vivo. In contrast, activation of PPARα increased only AdipoR2 in cultured cells. This result is consistent with previous studies that found PPARα 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 PPARα may be involved in the regulation of AdipoR2 expression. Activation of PPARγ 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 PPARγ activation. The increase of AdipoR2 expression in adipocytes may be caused by the secondary effect on adipocyte differentiation by PPARγ 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 PPARα 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 PPARα activation. Further studies using AdipoR knockout mice will be required to clarify this point. We also showed that activation of PPARγ or food restriction increased the ratio of HMW to total adiponectin and that activation of PPARα 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 PPARγ 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 PPARα and -γ can improve obesity-induced insulin resistance. First, activation of PPARα suppresses inflammation and adipose hypertrophy in WAT. Second, dual activation of PPARα and -γ enhances the action of adiponectin by increasing AdipoR expression and the ratio of HMW to total adiponectin.

ACKNOWLEDGMENTS

This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan; a grant from the Human Science Foundation (to T.K.); a grant-in-aid for the development of innovative technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.K.); Grant-in Aid for Creative Scientific Research 10NP0201 from the Japan Society for the Promotion of Science (to T.K.); and health science research grants (research on human genome and gene therapy) from the Ministry of Health, Labor and Welfare of Japan (to T.K.).

We are grateful to A. Itoh and A. Okano for their excellent technical assistance.

REFERENCES

1. Kersten S, Desvergne B, Wahli W: Roles of PPARs in health and disease. *Nature* 405:421–424, 2000
2. Lowell BB: PPARγ: an essential regulator of adipogenesis and modulator of fat cell function. *Cell* 99:239–242, 1999
3. Spiegelman BM, Flier JS: Adipogenesis and obesity: rounding out the big picture. *Cell* 87:377–389, 1996
4. Gonzalez FJ: Recent update on the PPAR α-null mouse. *Biochimie* 79:139–144, 1997
5. Auwerx J: PPARγ, the ultimate thrifty gene. *Diabetologia* 42:1033–1049, 1999
6. Evans RM, Barish GD, Wang YX: PPARs and the complex journey to obesity. *Nat Med* 10:355–361, 2004
7. Yamauchi T, Kamon J, Waki H, Murakami K, Motojima K, Komeda K, Ide T, Kubota N, Terauchi Y, Tobe K, Miki H, Tsuchida A, Akanuma Y, Nagai R, Kimura S, Kadowaki T: The mechanisms by which both heterozygous peroxisome proliferator-activated receptor γ (PPARγ) deficiency and PPARγ agonist improve insulin resistance. *J Biol Chem* 276:41245–41254, 2001
8. Yamauchi T, Waki H, Kamon J, Murakami K, Motojima K, Komeda K, Miki H, Kubota N, Terauchi Y, Tsuchida A, Tsuboyama-Kasaoka N, Yamauchi N, Ide T, Hori W, Kato S, Fukayama M, Akanuma Y, Ezaki O, Itai A, Nagai R, Kimura S, Tobe K, Kagechika H, Shudo K, Kadowaki T: Inhibition of RXR and PPARγ ameliorates diet-induced obesity and type 2 diabetes. *J Clin Invest* 108:1001–1013, 2001
9. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7:941–946, 2001
10. Guerre-Millo M, Gervois P, Raspe E, Madsen L, Poulain P, Derudas B, Herbert JM, Winegar DA, Willson TM, Fruchart JC, Berge RK, Staels B: Peroxisome proliferator-activated receptor α activators improve insulin sensitivity and reduce adiposity. *J Biol Chem* 275:6638–16642, 2000
11. Ye JM, Doyle PJ, Iglesias MA, Watson DG, Cooney GJ, Kraegen EW: Peroxisome proliferator-activated receptor (PPAR)-α activation lowers muscle lipids and improves insulin sensitivity in high fat-fed rats: comparison with PPAR-γ activation. *Diabetes* 50:411–417, 2001
12. Chou CJ, Haluzik M, Gregory C, Dietz KR, Vinson C, Gavrilova O, Reitman ML: WY14,643, a peroxisome proliferator-activated receptor alpha (PPARα) agonist, improves hepatic and muscle steatosis and reverses insulin resistance in lipoatrophic A-ZIP/F-1 mice. *J Biol Chem* 277:24484–24489, 2002
13. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808, 2003
14. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821–1830, 2003
15. Pickup JC, Mattock MB, Chusney GD, Burt D: NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* 40:1286–1292, 1997
16. Yudkin JS, Stehouwer CD, Emeis JJ, Coppack SW: C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* 19:972–978, 1999
17. Festa A, D'Agostino R Jr, Howard G, Mykkanen L, Tracy RP, Haffner SM: Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation* 102:42–47, 2000
18. Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL, Sundseth SS, Winegar DA, Blanchard DE, Spencer TA, Willson TM: Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J Biol Chem* 270:12953–12956, 1995
19. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T: Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423:762–769, 2003
20. Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, Yano W, Froguel P, Nagai R, Kimura S, Kadowaki T, Noda T: Disruption of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem* 277:25863–25866, 2002
21. Motojima K, Passilly P, Peters JM, Gonzalez FJ, Latruffe N: Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor α and γ activators in a tissue- and inducer-specific manner. *J Biol Chem* 273:16710–16714, 1998
22. Chao L, Marcus-Samuels B, Mason MM, Moitra J, Vinson C, Arioglu E, Gavrilova O, Reitman ML: Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. *J Clin Invest* 106:1221–1228, 2000
23. Moore GB, Chapman H, Holder JC, Lister CA, Piercy V, Smith SA, Clapham

JC: Differential regulation of adipocytokine mRNAs by rosiglitazone in db/db mice. *Biochem Biophys Res Commun* 286:735–741, 2001
24. Kim H, Haluzik M, Asghar Z, Yau D, Joseph JW, Fernandez AM, Reitman ML, Yakar S, Stannard B, Heron-Milhavet L, Wheeler MB, LeRoith D: Peroxisome proliferator–activated receptor-α agonist treatment in a transgenic model of type 2 diabetes reverses the lipotoxic state and improves glucose homeostasis. *Diabetes* 52:1770–1778, 2003
25. Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R, Kadowaki T: Impaired multimerization of human adiponectin mutants associated with diabetes: molecular structure and multimer formation of adiponectin. *J Biol Chem* 278:40352–40363, 2003
26. Li AC, Binder CJ, Gutierrez A, Brown KK, Plotkin CR, Pattison JW, Valledor AF, Davis RA, Willson TM, Witztum JL, Palinski W, Glass CK: Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARα, β/δ, and γ. *J Clin Invest* 114:1564–1576, 2004
27. Barbera MJ, Schluter A, Pedraza N, Iglesias R, Villarroya F, Giralt M: Peroxisome proliferator-activated receptor α activates transcription of the brown fat uncoupling protein-1 gene: a link between regulation of the thermogenic and lipid oxidation pathways in the brown fat cell. *J Biol Chem* 276:1486–1493, 2001
28. Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez De Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, Piomelli D: Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. *Nature* 425:90–93, 2003
29. Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W: Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -β, and -γ in the adult rat. *Endocrinology* 137:354–366, 1996
30. Auboeuf D, Rieusset J, Fajas L, Vallier P, Frering V, Riou JP, Staels B, Auwerx J, Laville M, Vidal H: Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-α in humans: no alteration in adipose tissue of obese and NIDDM patients. *Diabetes* 46:1319–1327, 1997
31. Sartipy P, Loskutoff DJ: Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 100:7265–7270, 2003
32. Tsuchida A, Yamauchi T, Ito Y, Hada Y, Maki T, Takekawa S, Kamon J, Kobayashi M, Suzuki R, Hara K, Kubota N, Terauchi Y, Froguel P, Nakae J, Kasuga M, Accili D, Tobe K, Ueki K, Nagai R, Kadowaki T: Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. *J Biol Chem* 279:30817–30822, 2004
33. Crouch E, Persson A, Chang D, Heuser J: Molecular structure of pulmonary surfactant protein D (SP-D). *J Biol Chem* 269:17311–17319, 1994
34. McCormack FX, Pattanajitvilai S, Stewart J, Possmayer F, Inchley K, Voelker DR: The Cys6 intermolecular disulfide bond and the collagen-like region of rat SP-A play critical roles in interactions with alveolar type II cells and surfactant lipids. *J Biol Chem* 272:27971–27979, 1997
35. Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE: Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem* 279:12152–12162, 2004
36. Flier JS: Obesity wars: molecular progress confronts an expanding epidemic. *Cell* 116:337–350, 2004
37. Kelly LJ, Vicario PP, Thompson GM, Candelore MR, Doebber TW, Ventre J, Wu MS, Meurer R, Forrest MJ, Conner MW, Cascieri MA, Moller DE: Peroxisome proliferator-activated receptors γ and α mediate in vivo regulation of uncoupling protein (UCP-1, UCP-2, UCP-3) gene expression. *Endocrinology* 139:4920–4927, 1998
38. Armstrong MB, Towle HC: Polyunsaturated fatty acids stimulate hepatic UCP-2 expression via a PPARα-mediated pathway. *Am J Physiol* 281: E1197–E1204, 2001
39. Granneman JG, Lahners KN, Chaudhry A: Molecular cloning and expression of the rat β3-adrenergic receptor. *Mol Pharmacol* 40:895–899, 1991
40. Atgie C, D'Allaire F, Bukowiecki LJ: Role of β1- and β3-adrenoceptors in the regulation of lipolysis and thermogenesis in rat brown adipocytes. *Am J Physiol* 273:C1136–C1142, 1997
41. Guzman M, Lo Verme J, Fu J, Oveisi F, Blazquez C, Piomelli D: Oleoylethanolamide stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activated receptor α (PPAR-α). *J Biol Chem* 279:27849–27854, 2004
42. Reifel-Miller A, Otto K, Hawkins E, Barr R, Bensch WR, Bull C, Dana S, Klausing K, Martin JA, Rafaeloff-Phail R, Rafizadeh-Montrose C, Rhodes G, Robey R, Rojo I, Rungta D, Snyder D, Wilbur K, Zhang T, Zink R, Warshawsky A, Brozinick JT: A peroxisome proliferator-activated receptor α/γ dual agonist with a unique in vitro profile and potent glucose and lipid effects in rodent models of type 2 diabetes and dyslipidemia. *Mol Endocrinol* 19:1593–1605, 2005
43. Staels B, Koenig W, Habib A, Merval R, Lebret M, Torra IP, Delerive P, Fadel A, Chinetti G, Fruchart JC, Najib J, Maclouf J, Tedgui A: Activation of human aortic smooth-muscle cells is inhibited by PPARα but not by PPARγ activators. *Nature* 393:790–793, 1998
44. Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, Gonzalez FJ, Fruchart JC, Tedgui A, Haegeman G, Staels B: Peroxisome proliferator-activated receptor α negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-κB and AP-1. *J Biol Chem* 274:32048–54, 1999
45. Chinetti G, Zawadski C, Fruchart JC, Staels B: Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors PPARα, PPARγ, and LXR. *Biochem Biophys Res Commun* 314:151–158, 2004
46. Ajuwon KM, Spurlock ME: Adiponectin inhibits lipopolysaccharide-induced NF-κB activation and IL-6 production and increases PPARγ2 expression in adipocytes. *Am J Physiol Regul Integr Comp Physiol* 288:R1220–R1225, 2005

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 13, pp. 8748–8755, March 31, 2006

Pioglitazone Ameliorates Insulin Resistance and Diabetes by Both Adiponectin-dependent and -independent Pathways*

Received for publication, May 23, 2005, and in revised form, December 7, 2005 Published, JBC Papers in Press, January 23, 2006, DOI 10.1074/jbc.M505649200

Naoto Kubota[‡§¶], Yasuo Terauchi[‡§], Tetsuya Kubota[‡¶], Hiroki Kumagai[‡], Shinsuke Itoh[‡], Hidemi Satoh[‡], Wataru Yano[‡], Hitomi Ogata[‖], Kumpei Tokuyama[‖], Iseki Takamoto[‡§¶], Tomoka Mineyama[‡], Michiro Ishikawa[**], Masao Moroi[**], Kaoru Sugi[**], Toshimasa Yamauchi[‡§], Kohjiro Ueki[‡], Kazuyuki Tobe[‡§], Tetsuo Noda[‡‡§§], Ryozo Nagai[¶¶], and Takashi Kadowaki[‡§¶1]

From the [‡]Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, the [§]CREST of Japan Science and Technology Agency, Saitama, 332-0012, the [¶]Division of Applied Nutrition, National Institute of Health and Nutrition, Tokyo 162-8636, the [‖]Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba 305-0006, the [**]Division of Cardiovascular Medicine, Toho University, Ohashi Hospital, Tokyo 153-8515, the [‡‡]Department of Cell Biology, Japanese Foundation for Cancer Research-Cancer Institute, Tokyo 135-8550, the [§§]Department of Molecular Genetics, Tohoku University School of Medicine, Sendai 980-8575, and the [¶¶]Department of Cardiovascular Diseases, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan

Thiazolidinediones have been shown to up-regulate adiponectin expression in white adipose tissue and plasma adiponectin levels, and these up-regulations have been proposed to be a major mechanism of the thiazolidinedione-induced amelioration of insulin resistance linked to obesity. To test this hypothesis, we generated adiponectin knock-out (*adipo*$^{-/-}$) ob/ob mice with a C57B/6 background. After 14 days of 10 mg/kg pioglitazone, the insulin resistance and diabetes of ob/ob mice were significantly improved in association with significant up-regulation of serum adiponectin levels. Amelioration of insulin resistance in ob/ob mice was attributed to decreased glucose production and increased AMP-activated protein kinase in the liver but not to increased glucose uptake in skeletal muscle. In contrast, insulin resistance and diabetes were not improved in *adipo*$^{-/-}$ob/ob mice. After 14 days of 30 mg/kg pioglitazone, insulin resistance and diabetes of ob/ob mice were again significantly ameliorated, which was attributed not only to decreased glucose production in the liver but also to increased glucose uptake in skeletal muscle. Interestingly, *adipo*$^{-/-}$ob/ob mice also displayed significant amelioration of insulin resistance and diabetes, which was attributed to increased glucose uptake in skeletal muscle but not to decreased glucose production in the liver. The serum-free fatty acid and triglyceride levels as well as adipocyte sizes in ob/ob and *adipo*$^{-/-}$ob/ob mice were unchanged after 10 mg/kg pioglitazone but were significantly reduced to a similar degree after 30 mg/kg pioglitazone. Moreover, the expressions of TNFα and resistin in adipose tissues of ob/ob and *adipo*$^{-/-}$ob/ob mice were unchanged after 10 mg/kg pioglitazone but were decreased after 30 mg/kg pioglitazone. Thus, pioglitazone-induced amelioration of insulin resistance and diabetes may occur adiponectin dependently in the liver and adiponectin independently in skeletal muscle.

Thiazolidinediones (TZDs)[2] have been shown to act as insulin sensitizers in animal models of obesity-linked insulin resistance and diabetes, and they have been widely used as therapeutic agents for the treatment of type 2 diabetes (1–5). TZDs have been proposed to ameliorate insulin resistance by binding to and activating peroxisome proliferator-activated receptor γ (PPARγ) in adipose tissue, thereby promoting adipose differentiation and increasing the number of small adipocytes that are more sensitive to insulin (6–9). Generation of small insulin-sensitive adipocytes by TZDs appears to be associated with amelioration of insulin resistance (10, 11). TZDs also lower circulating serum triglyceride and free fatty acid levels and down-regulate the production and secretion of TNFα and resistin (6, 10–15).

Adiponectin is an adipose tissue-derived secreted protein that circulates in plasma (16–19). We previously reported (20) finding that replenishment of adiponectin ameliorated insulin resistance in obese mice with decreased plasma adiponectin levels and that a combination of physiological doses of adiponectin and leptin reversed insulin resistance in lipoatrophic mice. Independently, administration of adiponectin has been reported to decrease plasma glucose levels by suppressing hepatic glucose production (21, 22), and administration of globular adiponectin reportedly lowers elevated fatty acid concentrations by oxidizing fatty acids in muscle (23). We and others (24, 25) have also demonstrated that adiponectin-deficient (*adipo*$^{-/-}$) mice are insulin-resistant and glucose-intolerant. Previous studies have shown that adiponectin stimulates fatty acid oxidation in skeletal muscle and inhibits glucose production in the liver by activating AMP-activated protein kinase (AMPK) (26) through its specific receptors, AdipoR1 and AdipoR2 (27). As a result, adiponectin has come to be recognized as a major insulin-sensitizing hormone (28, 29).

TZDs increase plasma adiponectin levels in animal models of obesity and diabetes, nondiabetic subjects, and patients with type 2 diabetes, and the improvement in insulin sensitivity in response to TZD administration is associated with an increase in circulating adiponectin (20, 30–35). Thus, it is reasonable to speculate that the action whereby TZDs increase insulin sensitivity is mediated, at least in part, by increased adiponectin. However, whether the TZD-induced increase in

* This work was supported by a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan, a grant-in-aid for the Development of Innovative Technology from the Ministry of Education, Culture, Sports, Science and Technology (to T. K.), and by Health Science Research Grants (Research on Human Genome and Gene Therapy) from the Ministry of Health and Welfare (to T. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: 81-3-5800-8815; Fax: 81-3-5800-9797; E-mail: kadowaki-3im@h.u-tokyo.ac.jp.

[2] The abbreviations used are: TZD, thiazolidinedione; PPARγ, peroxisome proliferator-activated receptor γ; AMPK, AMP-activated protein kinase; EGP, endogenous glucose production; GIR, glucose infusion rate(s); PEPCK, phosphoenolpyruvate carboxykinase; OGTT, oral glucose tolerance test; AUC, area under the curve; TG, triglyceride; FFA, free fatty acid; TNFα, tumor necrosis factor α.

plasma adiponectin is causally involved in TZD-mediated insulin-sensitizing effects has not been addressed experimentally.

To address this issue, in the present study, we used *adipo*$^{-/-}$ob/ob mice with a C57Bl/6 background to investigate whether the PPARγ agonist pioglitazone is capable of ameliorating insulin resistance in the absence of adiponectin. The absence of adiponectin had no effect on either the obesity or the diabetic phenotype of these mice. We found that the insulin resistance and diabetes of ob/ob mice was significantly improved in association with significant up-regulation of serum adiponectin levels after 14 days of 10 mg/kg pioglitazone treatment. Amelioration of insulin resistance in ob/ob mice was attributed to decreased glucose production and increased AMPK in the liver but not to increased glucose uptake in skeletal muscle. In contrast, insulin resistance and diabetes were not improved in *adipo*$^{-/-}$ob/ob mice. After 14 days of 30 mg/kg pioglitazone treatment, insulin resistance and diabetes of ob/ob mice were again significantly ameliorated, which was attributed not only to decreased glucose production in the liver but also to increased glucose uptake in skeletal muscle. Interestingly, *adipo*$^{-/-}$ob/ob mice also displayed significant amelioration of insulin resistance and diabetes, which was attributed to increased glucose uptake in skeletal muscle but not to decreased glucose production in the liver. Thus, pioglitazone-induced amelioration of insulin resistance and diabetes is mediated via both adiponectin-dependent pathway in the liver and adiponectin-independent pathway in skeletal muscle.

EXPERIMENTAL PROCEDURES

Animals and Genotyping

Mice were housed on a 12-h light-dark cycle and fed standard chow CE-2 (CLEA Japan Inc., Tokyo, Japan) with the following composition: 25.6% (w/w) protein, 3.8% fiber, 6.9% ash, 50.5% carbohydrates, 4% fat, and 9.2% water. To rule out the potential impact of the expression cassettes for the selection of targeted ES cells in the targeted allele on the expression of genes surrounding the adiponectin locus, selection cassettes were deleted by the Cre-Pac method as described previously (36), with some modification. We then backcrossed the original *adipo*$^{-/-}$ mice (C57Bl/6 and 129/sv background) (24) with C57Bl/6 mice more than seven times. ob/ob and *adipo*$^{-/-}$ob/ob mice were prepared by *adipo*$^{+/-}$ob/+ mouse intercrosses. All experiments in this study were conducted on male littermates. The animal care and procedures of the experiments were approved by the Animal Care Committee of the University of Tokyo.

Pioglitazone Treatment Study

10 mg/kg pioglitazone (AD-4833-HCl) or vehicle (0.25% carboxymethylcellulose) was adnimistered to ob/ob and *adipo*$^{-/-}$ob/ob mice by oral gavage once daily for 14 consecutive days. 30 mg/kg pioglitazone or vehicle was also adnimistered to ob/ob and *adipo*$^{-/-}$ob/ob mice by oral gavage once daily for 14 consecutive days. Pioglitazone was kindly provided by Takeda Chemical Industries Co., Ltd. (Osaka, Japan).

Hyperinsulinemic-Euglycemic Clamp Study

Clamp studies were carried out as described previously (37) with slight modifications. In brief, 2–3 days before the study, an infusion catheter was inserted into the right jugular vein under general anesthesia with sodium pentobarbital. Studies were performed on mice under conscious and unstressed conditions after a 6-h fast. A primed continuous infusion of insulin (Humulin R, Lilly) was given (5.0 milliunits/kg/min), and the blood glucose concentration, monitored every 5 min, was maintained at ~120 mg/dl by administration of glucose (5 g of glucose per 10 ml enriched to ~20% with [6,6-$^2H_2$]glucose (Sigma)) for 120 min. Blood was sampled via tail tip bleeds at 90, 105, and 120 min for determination of the rate of glucose disappearance (R_d). R_d was calculated according to nonsteady-state equations (37), and endogenous glucose production (EGP) was calculated as the difference between R_d and exogenous glucose infusion rates (GIR) (37).

Western Blot Analysis

Tissues were excised and homogenized in ice-cold buffer A (25 mM Tris-HCl (pH 7.4), 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). Samples were separated on polyacrylamide gels and transferred to a Hybond-P polyvinylidene difluoride transfer membrane (Amersham Biosciences). Bands were detected with ECL detection reagents (Amersham Biosciences). To examine AMPK phosphorylation and protein levels, hepatic lysates were blotted for anti-phosho-AMPK (Cell Signaling Technology, Inc., Beverly, MA) and anti-AMPK (Cell Signaling Technology, Inc.) antibody.

Serum Adiponectin and Lipid Measurements

Mice were fasted for more than 16 h before the measurements. Serum adiponectin levels were determined with a mouse adiponectin enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Serum triglyceride and free fatty acids (Wako Pure Chemical Industries Ltd., Osaka, Japan) were assayed by enzymatic methods.

In Vivo Glucose Homeostasis

Glucose Tolerance Test—Mice were fasted for more than 16 h before the study and then orally loaded with glucose, 1.5 mg/g body weight. Blood samples were collected from the orbital sinus at different times, and glucose was measured with an automatic glucometer (Glutest Ace, Sanwa Chemical Co., Nagoya, Japan). Whole blood was collected and centrifuged in heparinized tubes, and the plasma was stored at −20 °C. Insulin levels were determined with an insulin radioimmunoassay kit (Biotrak, Amersham Biosciences, Buckinghamshire, UK) with rat insulin as the standard (38).

Insulin Tolerance Test—Mice were given free access to food and then fasted during the study. They were intraperitoneally challenged with human insulin, 0.75 milliunits/g of body weight (Humulin R), and venous blood samples were drawn at different times (38). The changes were plotted as a percentage of basal glucose *versus* time.

Measurement of Adipocyte Size

Epididymal white adipose tissue was routinely processed for paraffin embedding, and 2-μm sections were cut and mounted on silanized slides. The adipose tissue was stained with hematoxylin and eosin, and total adipocyte area was manually traced and analyzed with Win ROOF software (Mitani Co. Ltd., Chiba, Japan). White adipocyte area was measured in 200 or more cells per mouse in each group according to methods described previously (39), with slight modifications.

RNA Preparation and Taqman PCR

Total RNA was prepared from adipose tissue with an RNeasy Mini Kit (Qiagen Co., Düsseldorf, Germany) according to the manufacturer's instructions. mRNA levels in white adipose tissue were quantitatively analyzed by fluorescence-based reverse transcriptase-PCR. The reverse transcription mixture was amplified with specific primers, using an ABI

Prism 7000 sequence detector equipped with a thermocycler. The primers used for β-actin were as described previously (40). The primers used for phosphoenolpyruvate carboxykinase (PEPCK), TNFα, and resistin were purchased from Applied Biosystems (Foster City, CA). Relative expression levels were compared after normalization to β-actin.

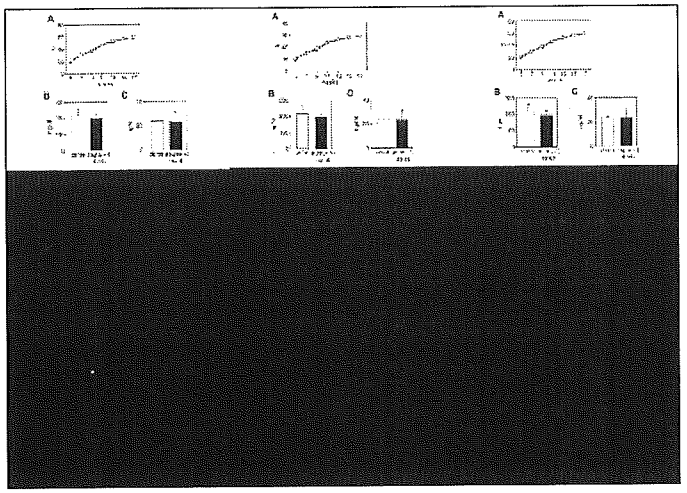

FIGURE 1. **Absence of adiponectin had no effects on obesity, fasting hyperglycemia, or fasting hyperinsulinemia.** *A–C*, body weights (*A*), fasting blood glucose (*B*), and fasting insulin (*C*) of ob/ob and *adipo*$^{-/-}$ob/ob mice. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open circles* and *bars*, $n = 9$) and *adipo*$^{-/-}$ob/ob mice (*closed squares* and *bars*, $n = 8$).

RESULTS

Absence of Adiponectin Had No Effects on Obesity, Fasting Hyperglycemia, or Fasting Hyperinsulinemia—The ob/ob mice and *adipo*$^{-/-}$ob/ob mice on a standard diet gained total body weight at comparable rates (Fig. 1*A*). Moreover, the ob/ob and *adipo*$^{-/-}$ob/ob mice showed comparable fasting hyperglycemia (Fig. 1*B*) and comparable fasting hyperinsulinemia (Fig. 1*C*). These data indicate that the absence of adiponectin had no effect on either the obesity or the diabetic phenotype of these mice.

10 mg/kg Pioglitazone for 14 Days Improved Diabetes in ob/ob Mice but Not in adipo$^{-/-}$*ob/ob Mice*—Ob/ob mice showed diabetic glucose tolerance (Fig. 2*A*). 10 mg/kg pioglitazone for 14 days significantly increased serum adiponectin levels in the ob/ob mice (Fig. 2*A*, *inset*). After 14 days of 10 mg/kg pioglitazone treatment, an oral glucose tolerance test (OGTT) showed that the blood glucose level of pioglitazone-treated ob/ob mice 15 min after glucose loading was significantly lower than that of untreated ob/ob mice (Fig. 2*A*). *Adipo*$^{-/-}$ob/ob mice showed comparable diabetic glucose tolerance to ob/ob mice (Fig. 2*B*). Serum adiponectin levels were not detectable in *adipo*$^{-/-}$ob/ob mice before and after 14 days of 10 mg/kg pioglitazone treatment (Fig. 2*B*, *inset*). Unlike ob/ob mice, the blood glucose levels before and after glucose loading were indistinguishable between untreated and treated *adipo*$^{-/-}$ob/ob mice (Fig. 2*B*). We calculated the area under the curve (AUC) during the OGTT to quantitate glucose intolerance. Before pioglitazone treatment, the AUCs were indistinguishable between ob/ob and *adipo*$^{-/-}$ob/ob mice (Fig. 2*C*). The AUCs became significantly

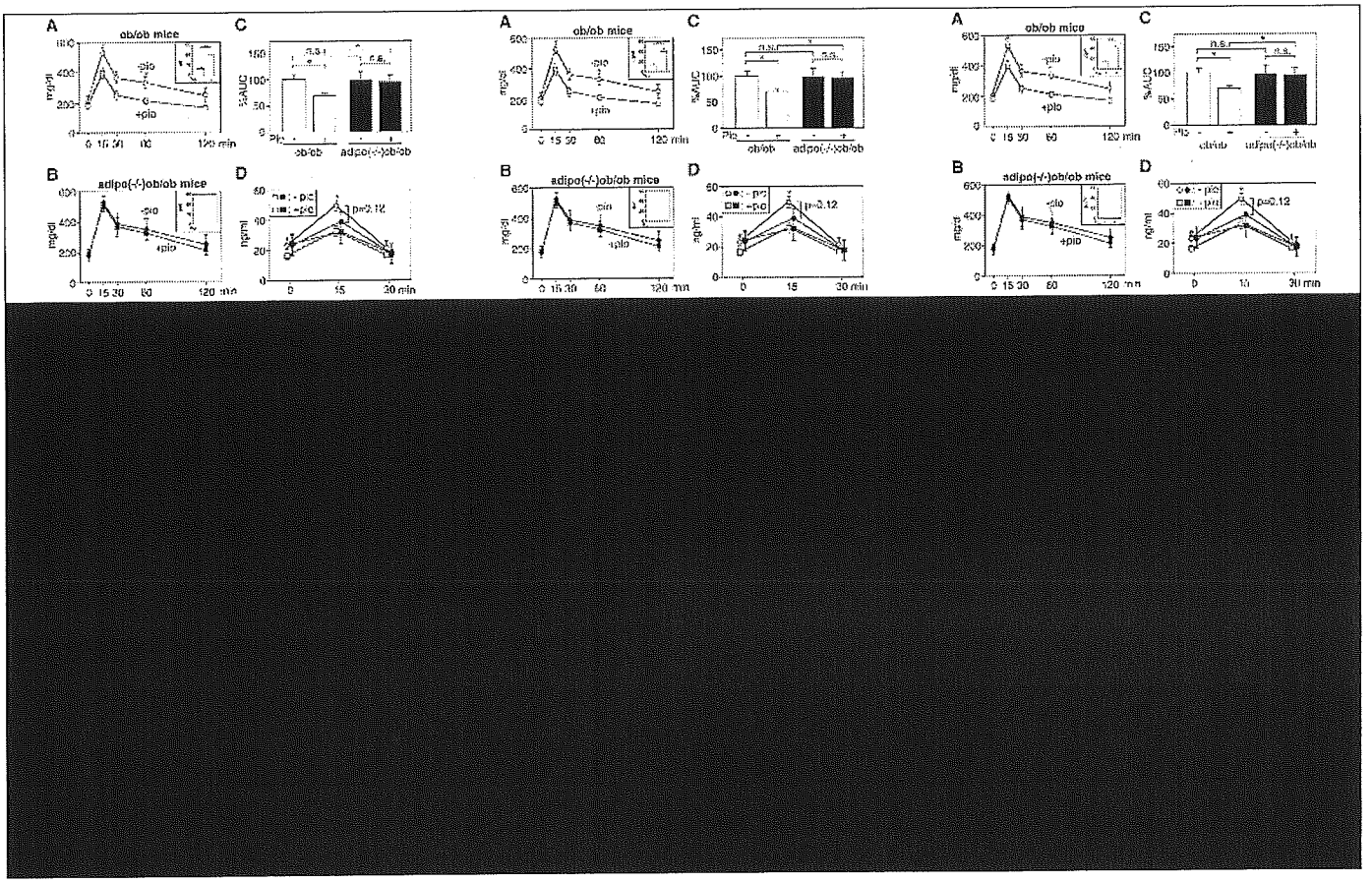

FIGURE 2. **10 mg/kg pioglitazone (*pio*) for 14 days improved diabetes in ob/ob mice but not in *adipo*$^{-/-}$ob/ob mice.** *A*, *B*, and *D*, blood glucose (*A*, *B*) and plasma insulin (*D*) levels during OGTT of ob/ob (*A*, *D*) and *adipo*$^{-/-}$ob/ob (*B*, *D*) mice not treated (*open circles* (*A*, *D*) or *closed circles* (*B*, *D*)) or treated (*open squares* (*A*, *D*) or *closed squares* (*B*, *D*)) with pioglitazone. *Insets* of *A* and *B* indicate serum adiponectin levels of ob/ob (*A*, *inset*) and *adipo*$^{-/-}$ob/ob (*B*, *inset*) mice not treated or treated with 10 mg/kg pioglitazone for 14 days. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open circles* and *squares*, $n = 7$) and *adipo*$^{-/-}$ob/ob mice (*closed circles*, *squares*, and *bars*, $n = 7–8$). *, $p < 0.05$. *C*, AUCs of the OGTT for *A* and *B*. Values are means ± S.E. *, $p < 0.05$; **, $p < 0.01$. *n.s.*, not significant.

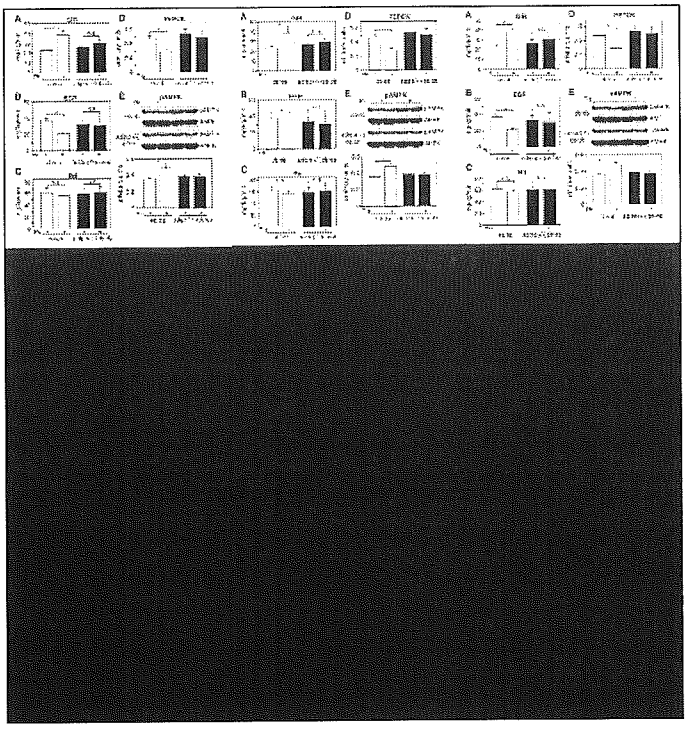

FIGURE 3. **10 mg/kg pioglitazone (*Pio*) for 14 days improved insulin resistance in the liver of ob/ob mice but not of *adipo*$^{-/-}$ob/ob mice.** *A–C*, GIR (*A*), EGP (*B*), and rates of R_d (*Rd*) (*C*) of ob/ob and *adipo*$^{-/-}$ob/ob mice in the clamp study. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 8–15$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 5–7$). *, $p < 0.05$. *D*, PEPCK expression levels in the livers of ob/ob and *adipo*$^{-/-}$ob/ob mice after the clamp studies. Relative expressions were compared after normalization to β-actin. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 8–15$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 5–7$). *, $p < 0.05$. *E*, phosphorylations of AMPK in the livers of ob/ob and *adipo*$^{-/-}$ob/ob mice after the clamp studies. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 4–5$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 4–5$). *, $p < 0.05$. *n.s.*, not significant.

smaller after pioglitazone treatment in the ob/ob/mice but not in the *adipo*$^{-/-}$ob/ob mice (Fig. 2*C*). Both ob/ob and *adipo*$^{-/-}$ob/ob mice showed hyperinsulinemia before and after glucose loading; however, the plasma insulin levels 15 min after glucose loading tended to be reduced in the *adipo*$^{-/-}$ob/ob mice as compared with ob/ob mice ($p = 0.11$), as we reported previously in the *adipo*$^{-/-}$ mice as compared with wild-type mice (24) (Fig. 2*D*). The plasma insulin levels of pioglitazone-treated ob/ob mice before and after glucose loading were significantly lower than those of untreated ob/ob mice (Fig. 2*D*). In contrast, the plasma insulin levels before and after glucose loading were indistinguishable between untreated and treated *adipo*$^{-/-}$ob/ob mice (Fig. 2*D*). These findings indicate that pioglitazone ameliorates diabetes in mice with an ob/ob background in an adiponectin-dependent manner.

10 mg/kg Pioglitazone for 14 Days Improved Insulin Resistance in the Liver of ob/ob Mice but Not of adipo$^{-/-}$*ob/ob Mice*—We next carried out hyperinsulinemic-euglycemic clamp studies in ob/ob and *adipo*$^{-/-}$ob/ob mice to investigate the effect of pioglitazone on amelioration of insulin resistance in the liver and skeletal muscle. Before pioglitazone treatment, GIR were comparable in ob/ob and *adipo*$^{-/-}$ob/ob mice (Fig. 3*A*). After 14 days of 10 mg/kg pioglitazone treatment, the GIR of pioglitazone-treated ob/ob mice was significantly higher than that of untreated ob/ob mice, indicating insulin resistance in ob/ob mice to be improved (Fig. 3*A*). In contrast, the GIR were indistinguishable between untreated and pioglitazone-treated *adipo*$^{-/-}$ob/ob mice (Fig. 3*A*). The amelioration of insulin resistance in ob/ob mice was, at least in part, due to decreased EGP (Fig. 3*B*). Rates of R_d were indistinguishable between ob/ob and *adipo*$^{-/-}$ob/ob mice, and 10 mg/kg pioglitazone for 14 days had no effect on these levels in either genotype (Fig. 3*C*). PEPCK expression levels in the liver were comparable in ob/ob and *adipo*$^{-/-}$ob/ob mice before pioglitazone treatment (Fig. 3*D*). 10 mg/kg pioglitazone for 14 days significantly decreased PEPCK expression in ob/ob, but not *adipo*$^{-/-}$ob/ob, mice (Fig. 3*D*). AMPK expression levels in the liver did not differ significantly between ob/ob and *adipo*$^{-/-}$ob/ob mice before or after pioglitazone treatment (Fig. 3*E*). AMPK activities before pioglitazone treatment were comparable in ob/ob and *adipo*$^{-/-}$ob/ob mice (Fig. 3*E*). AMPK phosphorylation in ob/ob mice was significantly increased after 10 mg/kg pioglitazone for 14 days but was unchanged in *adipo*$^{-/-}$ob/ob mice (Fig. 3*E*). These findings indicate that pioglitazone ameliorates hepatic, but not muscle, insulin resistance in mice with an ob/ob background in an adiponectin-dependent manner via, at least in part, decreased gluconeogenesis and increased AMPK activation.

30 mg/kg Pioglitazone for 14 Days Improved Diabetes to a Similar Degree in ob/ob and adipo$^{-/-}$*ob/ob Mice*—We next administered 30 mg/kg pioglitazone to ob/ob and *adipo*$^{-/-}$ob/ob mice for 14 days.

30 mg/kg pioglitazone also significantly increased serum adiponectin levels in the ob/ob mice (Fig. 4*A*, *inset*), but the serum adiponectin levels after 30 mg/ml pioglitazone were not significantly different from those after 10 mg/kg pioglitazone (Fig. 2*A*, *inset*, and Fig. 4A, *inset*). The blood glucose levels of pioglitazone-treated ob/ob mice before and after glucose loading were significantly lower than those of untreated ob/ob mice (Fig. 4*A*). Interestingly, the blood glucose levels of pioglitazone-treated *adipo*$^{-/-}$ob/ob mice before and after glucose loading became significantly lower than those of untreated *adipo*$^{-/-}$ob/ob mice, being similar to the levels seen in ob/ob/mice (Fig. 4*B*). The AUCs during the OGTT of both groups became smaller after pioglitazone treatment, but they were indistinguishable between the ob/ob and *adipo*$^{-/-}$ob/ob mice (Fig. 4*C*). The plasma insulin levels of pioglitazone-treated ob/ob mice before and after glucose loading were significantly lower than those of untreated ob/ob mice (Fig. 4*D*). Similarly, the plasma insulin levels before glucose loading of pioglitazone-treated *adipo*$^{-/-}$ob/ob mice also became significantly lower than those of untreated *adipo*$^{-/-}$ob/ob mice (Fig. 4*E*).

30 mg/kg Pioglitazone for 14 Days Improved Insulin Resistance in the Liver and Skeletal Muscle of ob/ob Mice but Only in Skeletal Muscle of adipo$^{-/-}$*ob/ob Mice*—We next carried out hyperinsulinemic-euglycemic clamp studies in ob/ob and *adipo*$^{-/-}$ob/ob mice to investigate the effect of 30 mg/kg pioglitazone treatment on amelioration of insulin resistance in the liver and skeletal muscle. After 14 days of 30 mg/kg pioglitazone treatment, the GIR of pioglitazone-treated ob/ob mice was significantly higher than that of untreated ob/ob mice (Fig. 5*A*). Interestingly, the GIR of pioglitazone-treated *adipo*$^{-/-}$ob/ob mice was also significantly higher than that of untreated *adipo*$^{-/-}$ob/ob mice, indicating insulin resistance in *adipo*$^{-/-}$ob/ob mice to be improved (Fig. 5*A*). The EGP was significantly decreased in ob/ob mice after 30 mg/kg pioglitazone treatment but not in *adipo*$^{-/-}$ob/ob mice (Fig. 5*B*). In contrast, the Rd was significantly increased in ob/ob and *adipo*$^{-/-}$ob/ob mice to a similar degree after 30 mg/kg pioglitazone treatment (Fig. 5*C*). 30 mg/kg pioglitazone significantly decreased PEPCK expression in ob/ob mice but not in *adipo*$^{-/-}$ob/ob mice (Fig. 5*D*). AMPK phosphorylation in ob/ob mice was significantly increased after 30 mg/kg pioglitazone for 14 days but was unchanged in *adipo*$^{-/-}$ob/ob mice (Fig. 5*E*). These findings suggest that the amelioration of insulin resistance in *adipo*$^{-/-}$ob/ob mice was, at least in part, due to increased glucose uptake in skeletal muscle.

30 mg/kg Pioglitazone for 14 Days, but Not 10 mg/kg for 14 Days, Ameliorated Adipocyte Hypertrophy in ob/ob and adipo$^{-/-}$*ob/ob Mice*—We previously demonstrated that TZDs increased the number of small

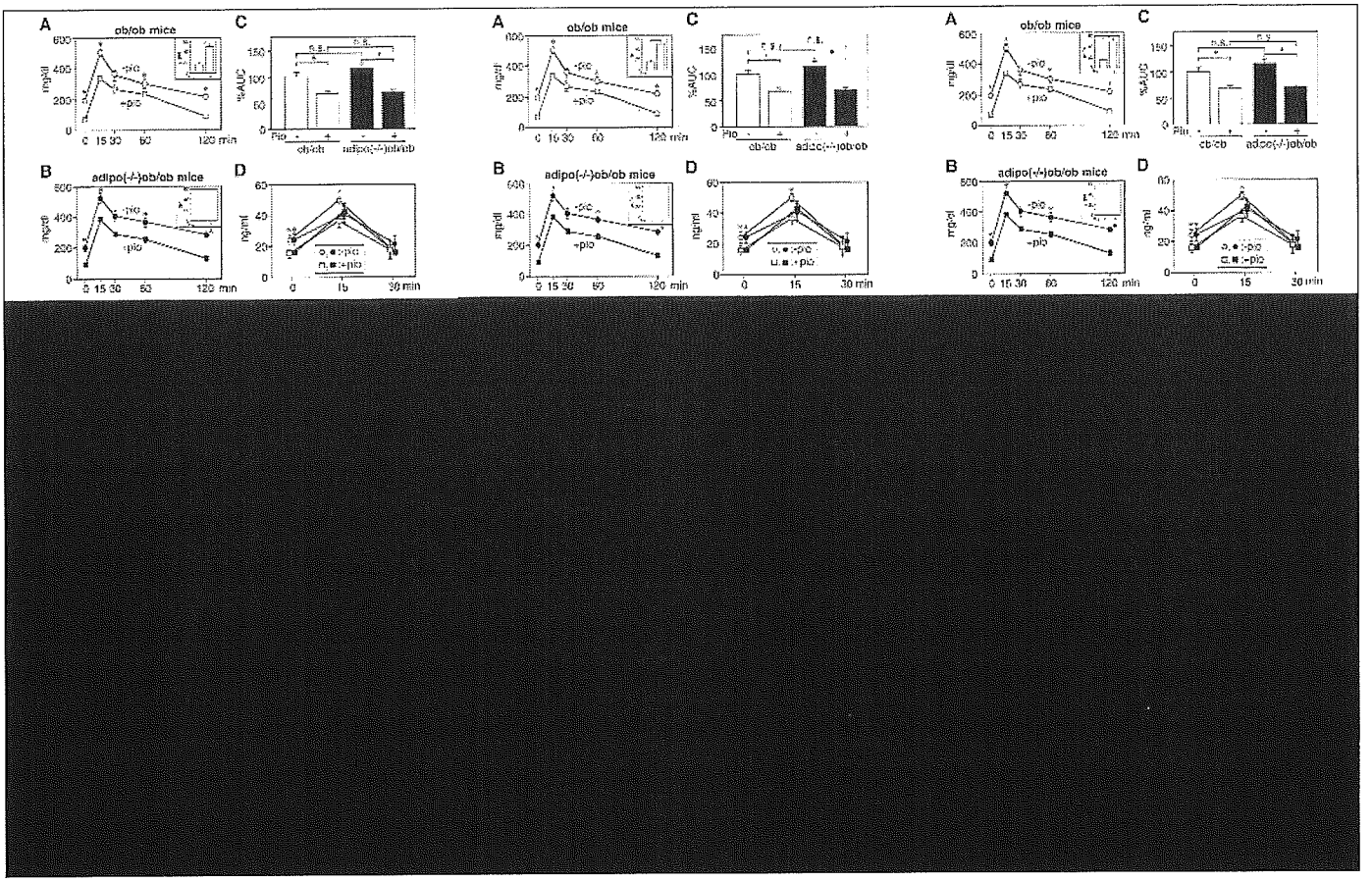

FIGURE 4. **30 mg/kg pioglitazone (*pio*) for 14 days improved diabetes to a similar degree in ob/ob and *adipo*$^{-/-}$ob/ob mice.** *A*, *B*, and *D*, blood glucose (*A*, *B*) and plasma insulin (*D*) levels during OGTT of ob/ob (*A*, *D*) and *adipo*$^{-/-}$ob/ob (*B*, *D*) mice not treated (*open circles* (*A*, *D*) or *closed circles* (*B*, *D*)) or treated (*open squares* (*A*, *D*) or *closed squares* (*B*, *D*)) with pioglitazone. *Insets* of *A* and *B* indicate serum adiponectin levels of ob/ob (*A*, *inset*) and *adipo*$^{-/-}$ob/ob (*B*, *inset*) mice not treated or treated with 30 mg/kg pioglitazone for 14 days. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open circles* and *squares*, $n = 8–9$) and *adipo*$^{-/-}$ob/ob mice (*closed circles*, *squares*, and *bars*, $n = 8$). *, $p < 0.05$. **, $p < 0.01$. *C*, AUCs of the OGTT for *A* and *B*. Values are means ± S.E. *, $p < 0.05$. *n.s.*, not significant.

adipocytes and decreased the number of large adipocytes, thereby ameliorating insulin resistance (6). To determine whether the presence of adiponectin is required for the reduction of average adipocyte size induced by TZDs to occur, we histologically analyzed epididymal fat pads after fixation and quantitation of adipocyte size. The adipocyte sizes of ob/ob and *adipo*$^{-/-}$ob/ob mice were indistinguishable and were not changed by 10 mg/kg pioglitazone for 14 days (Fig. 6, *A* and *B*). 30 mg/kg pioglitazone for 14 days, however, significantly reduced adipocyte sizes of ob/ob and *adipo*$^{-/-}$ob/ob mice to a similar degree (Fig. 6, *C* and *D*). These results suggest that pioglitazone can induce a reduction in adipocyte size in the absence of adiponectin or leptin or the absence of both.

30 mg/kg Pioglitazone, but Not 10 mg/kg, Significantly Decreased Serum Triglyceride and Free Fatty Acid Levels in ob/ob and adipo$^{-/-}$*ob/ob Mice*—In addition to improving insulin resistance, TZDs reportedly reduce serum triglyceride (TG) and free fatty acid (FFA) levels (6, 10–12). However, since the possible involvement of adiponectin in this action of TZDs remains unclear, we investigated the effects of pioglitazone treatment on serum lipid levels. The serum TG levels of the ob/ob and *adipo*$^{-/-}$ob/ob mice were essentially the same (Fig. 7*A*), and 10 mg/kg pioglitazone for 14 days did not change the serum TG levels in either genotype (Fig. 7*A*). Serum FFA levels were also indistinguishable between ob/ob and *adipo*$^{-/-}$ob/ob mice (Fig. 7*B*), and 10 mg/kg pioglitazone for 14 days again had no effect on these levels in either group of mice (Fig. 7*B*). However, 30 mg/kg pioglitazone for 14 days significantly decreased serum TG levels, to a similar degree, in ob/ob and *adipo*$^{-/-}$ob/ob mice (Fig. 7*C*). 30 mg/kg pioglitazone for 14 days also lowered FFA levels in both genotypes, and the serum FFA levels of ob/ob and *adipo*$^{-/-}$ob/ob mice became similar after pioglitazone treatment (Fig. 7*D*).

30 mg/kg Pioglitazone, but Not 10 mg/kg, Reduced TNFα and Resistin Expressions in ob/ob and adipo$^{-/-}$*ob/ob Mice*—TNFα and resistin have been shown to be important mediators of insulin resistance linked to obesity (13–15). TZDs reportedly reduce the expressions of TNFα and resistin (13–15), but whether adiponectin was involved in this action remains unclear. TNFα expression tended to be higher in the *adipo*$^{-/-}$ob/ob mice than in the ob/ob mice (Fig. 8*A*). After 14 days of 10 mg/kg pioglitazone treatment, TNFα expression was not significantly changed in either ob/ob or *adipo*$^{-/-}$ob/ob mice (Fig. 8*A*). Resistin expressions were indistinguishable between ob/ob and *adipo*$^{-/-}$ob/ob mice before and after 14 days of 10 mg/kg pioglitazone (Fig. 8*B*). After 14 days of 30 mg/kg pioglitazone, however, TNFα expressions were significantly decreased in both ob/ob and *adipo*$^{-/-}$ob/ob mice (Fig. 8*C*), and resistin expressions tended to be lower in both pioglitazone-treated groups than in the untreated groups (Fig. 8*D*).

DISCUSSION

TZDs have been reported to alleviate insulin resistance in adipose tissue, skeletal muscle, and the liver (5, 7–11). However, since PPARγ, which is bound and activated by TZDs, is predominantly expressed in adipose tissue, it is reasonable to speculate that the effect of TZDs on insulin resistance in skeletal muscle and the liver is mediated largely via the effects of TZDs on adipose tissue including alterations of adipokine expression and secretion by adipocytes (5, 7–11).

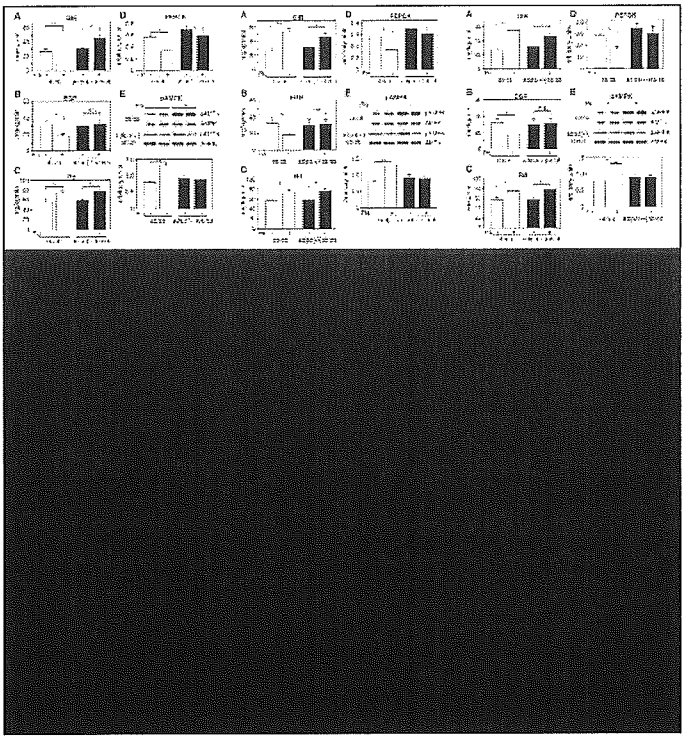

FIGURE 5. **30 mg/kg pioglitazone (*Pio*) for 14 days improved insulin resistance in the liver and skeletal muscle of ob/ob mice but only in skeletal muscle of *adipo*$^{-/-}$ob/ob mice.** *A–C*, GIR (*A*), EGP (*B*), and rates of R_d (*Rd*) (*C*) of ob/ob and *adipo*$^{-/-}$ob/ob mice in the clamp study. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 8–11$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 5–10$). *, $p < 0.05$. **, $p < 0.01$. *D*, PEPCK expression levels in the livers of ob/ob and *adipo*$^{-/-}$ob/ob mice after the clamp studies. Relative expressions were compared after normalization to β-actin. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 5–6$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 5–6$). *, $p < 0.05$. *E*, phosphorylations of AMPK in the livers of ob/ob and *adipo*$^{-/-}$ob/ob mice after the clamp studies. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 4$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 4$). **, $p < 0.01$. *n.s.*, not significant.

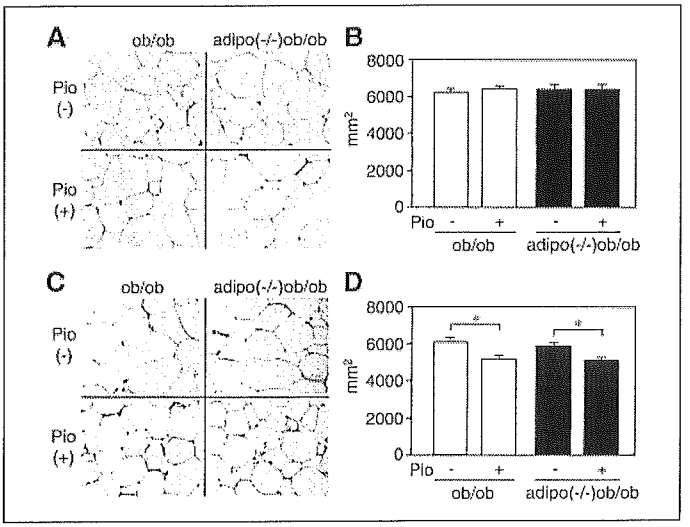

FIGURE 6. **30 mg/kg pioglitazone (*Pio*), but not 10 mg/kg, ameliorated adipocyte hypertrophy in ob/ob and *adipo*$^{-/-}$ob/ob mice.** Histological analyses (*A*, *C*; original magnification: ×100) and the sizes of adipocytes (*B*, *D*) of epididymal white adipose tissue in ob/ob and *adipo*$^{-/-}$ob/ob mice treated with 10 mg/kg pioglitazone for 14 days (*A*, *B*) or with 30 mg/kg pioglitazone for 14 days (*C*, *D*). Representative results are shown. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 7–8$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 7–8$) not treated or treated with pioglitazone. *, $p < 0.05$.

Adiponectin has been proposed to be a major insulin-sensitizing adipokine (20–25) and is a plausible candidate for one of the adipokines that may mediate the TZD-induced amelioration of insulin resistance.

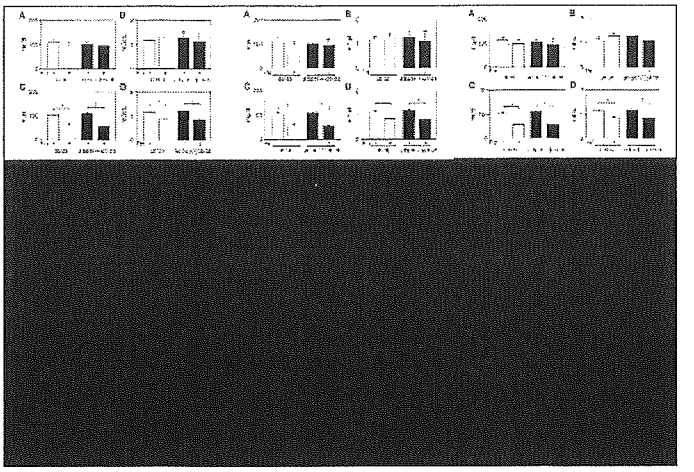

FIGURE 7. **30 mg/kg pioglitazone (*Pio*), but not 10 mg/kg, significantly decreased serum triglyceride and free fatty acid levels in ob/ob and *adipo*$^{-/-}$ob/ob mice.** Serum triglyceride (*A*, *C*) and free fatty acid (*B*, *D*) levels of ob/ob and *adipo*$^{-/-}$ob/ob mice treated with 10 mg/kg pioglitazone for 14 days (*A*, *B*) or with 30 mg/kg pioglitazone for 14 days (*C*, *D*). Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 7–8$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 7–8$). *, $p < 0.05$.

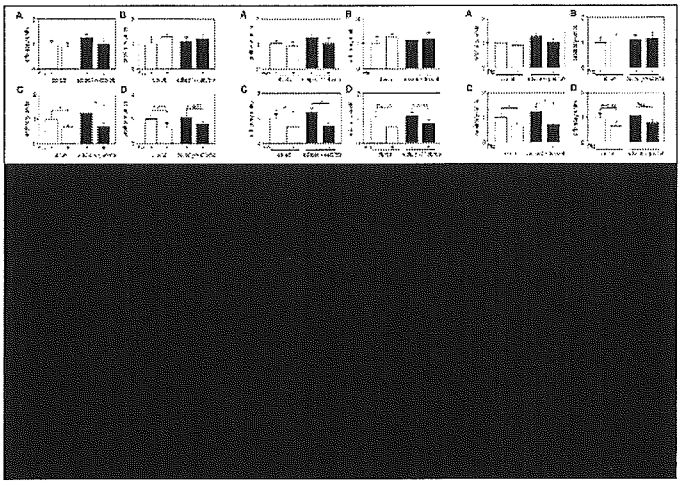

FIGURE 8. **30 mg/kg pioglitazone (*Pio*), but not 10 mg/kg, reduced TNFα and resistin expressions in ob/ob and *adipo*$^{-/-}$ob/ob mice.** TNFα (*A*, *C*) and resistin (*B*, *D*) expressions in white adipose tissues of ob/ob and *adipo*$^{-/-}$ob/ob mice treated with 10 mg/kg pioglitazone for 14 days (*A*, *B*) or with 30 mg/kg pioglitazone for 14 days (*C*, *D*). Relative expressions were compared after normalization to β-actin. Values are means ± S.E. of data obtained from the analysis of ob/ob mice (*open bars*, $n = 7–8$) and *adipo*$^{-/-}$ob/ob mice (*closed bars*, $n = 7–8$) not treated or treated with pioglitazone. *, $p < 0.05$.

Therefore, in this study, we used obesity models, ob/ob and *adipo*$^{-/-}$ob/ob mice, to investigate whether the TZD-induced increase in plasma adiponectin is causally involved in TZD-mediated insulin-sensitizing effects.

Insulin resistance and diabetes improved significantly in ob/ob mice in association with significant up-regulation of serum adiponectin levels with 14 days of 10 mg/kg pioglitazone treatment. Amelioration of insulin resistance in ob/ob mice was attributed to improvement of hepatic, but not muscle, insulin resistance. These improvements by pioglitazone were significantly obliterated in *adipo*$^{-/-}$ob/ob mice, indicating that adiponectin is causally involved in the 10 mg/kg pioglitazone-mediated amelioration of hepatic insulin resistance and diabetes. In fact, while PEPCK expression levels were significantly decreased and AMPK activity was significantly increased in the livers of ob/ob mice, these changes were not seen in mice without adiponectin. Interestingly, 10 mg/kg pioglitazone for 14 days failed to improve adipocyte hypertrophy, or the elevations of TG and FFA in serum and TNFα and resistin in white

adipose tissue, despite elevated serum adiponectin concentrations in ob/ob mice. This suggests that adiponectin-dependent amelioration of hepatic insulin resistance and diabetes occurs independently of adipocyte size, serum TG, and FFA levels or TNFα and resistin levels in adipose tissue.

On the other hand, 30 mg/kg pioglitazone for 14 days unexpectedly ameliorated insulin resistance and diabetes both in ob/ob and *adipo*$^{-/-}$ob/ob mice. Although the hepatic insulin resistance was not improved in *adipo*$^{-/-}$ob/ob mice as seen after 10 mg/kg pioglitazone treatment, muscle insulin resistance was alleviated in *adipo*$^{-/-}$ob/ob mice to a similar degree in ob/ob mice after 30 mg/kg pioglitazone for 14 days. This suggests that 30 mg/kg pioglitazone for 14 days can ameliorate muscle insulin resistance and diabetes via mechanisms which do not require the presence of adiponectin.

We previously reported that TZD treatment resulted in smaller adipocytes and a decreased number of large adipocytes, both of which were accompanied by decreases in TNFα, resistin, and FFA and an increase in adiponectin (6, 20). In fact, 30 mg/kg pioglitazone for 14 days, but not 10 mg/kg pioglitazone for 14 days, significantly reduced adipocyte size in ob/ob and *adipo*$^{-/-}$ob/ob mice to a similar degree. Moreover, insulin resistance-causing adipokines, such as TNFα, resistin, and FFA, were similarly reduced by 30 mg/kg pioglitazone for 14 days in *adipo*$^{-/-}$ob/ob mice as well as ob/ob mice. Thus, adiponectin was not absolutely required for 30 mg/kg pioglitazone-induced reductions in TNFα, resistin, or FFA. The smaller adipocytes, as well as the decreases in TNFα, resistin, and FFA, may have played a role in the amelioration of muscle insulin resistance and diabetes produced by 30 mg/kg pioglitazone for 14 days.

Recently, Wellen *et al.* (41) investigated whether the ability of TZDs to block TNFα action may be relevant to its ability to improve insulin action and the metabolism of lipids such as serum TG and FFA, using obese ob/ob mice lacking TNFα function. TZDs significantly ameliorated blood glucose and lipid levels in mice with and without TNFα function. This suggests that TZDs can improve blood glucose and lipid levels in a TNFα-independent manner. Therefore, resistin, FFA, and/or other cytokine(s), but perhaps not TNFα, may play roles in TZD-induced amelioration of insulin resistance and diabetes.

Although both low (10 mg/kg) and high (30 mg/kg) doses of pioglitazone ameliorated insulin resistance and diabetes, the underlying mechanisms may be different. The low dose of pioglitazone may be largely dependent on the adiponectin pathway, while the high dose of pioglitazone also improves adiponectin-independent pathways. High dose pioglitazone treatment in this study showed no significant difference in the ability to ameliorate insulin resistance and diabetes between ob/ob and *adipo*$^{-/-}$ob/ob mice, but the presence of adiponectin might have affected insulin resistance and diabetes in these mouse models, if the duration of 30 mg/kg pioglitazone treatment had been longer. The degree to which the adiponectin-dependent pathway is involved in TZD-induced amelioration of insulin resistance and diabetes merits further study in murine models and eventually in humans.

In this study, we addressed the important question of whether TZD-induced up-regulation of plasma adiponectin levels is causally involved in the insulin sensitizing actions of TZDs and demonstrated that TZD-induced amelioration of insulin resistance and diabetes may occur adiponectin-dependently in the liver and adiponectin-independently in skeletal muscle.

Acknowledgments—We thank Yoshinobu Sugitani, Shihoko Ito, Katsuko Takasawa, Hitomi Yamanaka, Eri Yoshida-Nagata, Ayumi Nagano, Ryuichi Taki, Miharu Nakashima, Namiko Kasuga, Yuko Miki, and Hiroshi Chiyonobu for their excellent technical assistance and animal care.

REFERENCES

1. Bowen, L., Stein, P. P., Stevenson, R., and Shulman, G. I. (1991) *Metabolism* 40, 1025–1030
2. Nolan, J. J., Ludvik, B., Beerdsen, P., Joyce, M., and Olefsky, J. (1994) *N. Engl. J. Med.* **331,** 1188–1193
3. Saltiel, A. R. (2001) *Cell* **104,** 517–529
4. Mauvais-Jarvis, F., and Kahn, C. R. (2000) *Diabetes Metab.* **26,** 433–448
5. Yki-Jarvinen, H. (2004) *N. Engl. J. Med.* **351,** 1106–1118
6. Okuno, A., Tamemoto, H., Tobe, K., Ueki, K., Mori, Y., Iwamoto, K., Umesono, K., Akanuma, Y., Fujiwara, T., Horikoshi, H., Yazaki, Y., and Kadowaki, T. (1998) *J. Clin. Invest.* **101,** 1354–1361
7. Evans, R. M., Barish, G. D., and Wang, Y. X. (2004) *Nat. Med.* **10,** 355–361
8. Rangwala, S. M., and Lazar, M. A. (2004) *Trends Pharmacol. Sci.* **25,** 331–336
9. Olefsky, J. M., and Saltiel, A. R. (2000) *Trends Endocrinol. Metab.* **11,** 362–368
10. Arner, P. (2003) *Trends Endocrinol. Metab.* **14,** 137–145
11. Moller, D. E. (2001) *Nature* **414,** 821–827
12. Yamauchi, T., Kamon, J., Waki, H., Murakami, K., Motojima, K., Komeda, K., Ide, T., Kubota, N., Terauchi, Y., Tobe, K., Miki, H., Tsuchida, A., Akanuma, Y., Nagai, R., Kimura, S., and Kadowaki, T. (2001) *J. Biol. Chem.* **276,** 41245–41254
13. Hotamisligil, G. S., Shargill, N. S., and Spiegelman, B. M. (1993) *Science* **259,** 87–91
14. Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., and Lazar, M. A. (2001) *Nature* **409,** 307–312
15. Kershaw, E. E., and Flier, J. S. (2004) *J. Clin. Endocrinol. Metab.* **89,** 2548–2556
16. Scherer, P. E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H. F. (1995) *J. Biol. Chem.* **270,** 26746–26749
17. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., and Matsubara, K. (1996) *Biochem. Biophys. Res. Commun.* **221,** 286–296
18. Hu, E., Liang, P., and Spiegelman, B. M. (1996) *J. Biol. Chem.* **271,** 10697–10703
19. Nakano, Y., Tobe, T., Choi-Miura, N. H., Mazda, T., and Tomita, M. (1996) *J. Biochem. (Tokyo)* **120,** 802–812
20. Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P., and Kadowaki, T. (2001) *Nat. Med.* **7,** 941–946
21. Berg, A. H., Combs, T. P., Du, X., Brownlee, M., and Scherer, P. E. (2001) *Nat. Med.* **7,** 947–953
22. Combs, T. P., Berg, A. H., Obici, S., Scherer, P. E., and Rossetti, L. (2001) *J. Clin. Invest.* **108,** 1875–1881
23. Fruebis, J., Tsao, T. S., Javorschi, S., Ebbets-Reed, D., Erickson, M. R., Yen, F. T., Bihain, B. E., and Lodish, H. F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 2005–2010
24. Kubota, N., Terauchi, Y., Yamauchi, T., Kubota, T., Moroi, M., Matsui, J., Eto, K., Yamashita, T., Kamon, J., Satoh, H., Yano, W., Froguel, P., Nagai, R., Kimura, S., Kadowaki, T., and Noda, T. (2002) *J. Biol. Chem.* **277,** 25863–25866
25. Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahashi, M., Arita, Y., Komuro, R., Ouchi, N., Kihara, S., Tochino, Y., Okutomi, K., Horie, M., Takeda, S., Aoyama, T., Funahashi, T., and Matsuzawa, Y. (2002) *Nat. Med.* **8,** 731–737
26. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kadowaki, T. (2002) *Nat. Med.* **8,** 1288–1295
27. Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N. H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., and Kadowaki, T. (2003) *Nature* **423,** 762–769
28. Pajvani, U. B., and Scherer, P. E. (2003) *Curr. Diab. Rep.* **3,** 207–213
29. Berg, A. H., Combs, T. P., and Scherer, P. E. (2002) *Trends Endocrinol. Metab.* **13,** 84–89
30. Combs, T. P., Wagner, J. A., Berger, J., Doebber, T., Wang, W. J., Zhang, B. B., Tanen, M., Berg, A. H., O'Rahilly, S., Savage, D. B., Chatterjee, K., Weiss, S., Larson, P. J., Gottesdiener, K. M., Gertz, B. J., Charron, M. J., Scherer, P. E., and Moller, D. E. (2002) *Endocrinology* **143,** 998–1007
31. Yu, J. G., Javorschi, S., Hevener, A. L., Kruszynska, Y. T., Norman, R. A., Sinha, M., and Olefsky, J. M. (2002) *Diabetes* **51,** 2968–2974
32. Chandran, M., Phillips, S. A., Ciaraldi, T., and Henry, R. R. (2003) *Diabetes Care* **26,** 2442–2450
33. Pajvani, U. B., Hawkins, M., Combs, T. P., Rajala, M. W., Doebber, T., Berger, J. P., Wagner, J. A., Wu, M., Knopps, A., Xiang, A. H., Utzschneider, K. M., Kahn, S. E., Olefsky, J. M., Buchanan, T. A., and Scherer, P. E. (2004) *J. Biol. Chem.* **279,** 12152–12162
34. Iwaki, M., Matsuda, M., Maeda, N., Funahashi, T., Matsuzawa, Y., Makishima, M., and Shimomura, I. (2003) *Diabetes* **52,** 1655–1663
35. Hirose, H., Kawai, T., Yamamoto, Y., Taniyama, M., Tomita, M., Matsubara, K.,

Okazaki, Y., Ishii, T., Oguma, Y., Takei, I., and Saruta, T. (2002) *Metabolism* **51,** 314–317
36. Taniguchi, M., Sanbo, M., Watanabe, S., Naruse, I., Mishina, M., and Yagi, T. (1998) *Nucleic Acids Res.* **26,** 679–680
37. Suzuki, R., Tobe, K., Aoyama, M., Inoue, A., Sakamoto, K., Yamauchi, T., Kamon, J., Kubota, N., Terauchi, Y., Yoshimatsu, H., Matsuhisa, M., Nagasaka, S., Ogata, H., Tokuyama, K., Nagai, R., and Kadowaki, T. (2004) *J. Biol.* Chem. **279,** 25039–25049
38. Kubota, N., Tobe, K., Terauchi, Y., Eto, K., Yamauchi, T., Suzuki, R., Tsubamoto, Y., Komeda, K., Nakano, R., Miki, H., Satoh, S., Sekihara, H., Sciacchitano, S., Lesniak, M., Aizawa, S., Nagai, R., Kimura, S., Akanuma, Y., Taylor, S. I., and Kadowaki, T. (2000) *Diabetes* **49,** 1880–1889
39. Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S., Nagai, R., Tobe, K., Kimura, S., and Kadowaki, T. (1999) *Mol. Cell* **4,** 597–609
40. Kubota, N., Terauchi, Y., Tobe, K., Yano, W., Suzuki, R., Ueki, K., Takamoto, I., Satoh, H., Maki, T., Kubota, T., Moroi, M., Okada-Iwabu, M., Ezaki, O., Nagai, R., Ueta, Y., Kadowaki, T., and Noda, T. (2004) *J. Clin. Invest.* **114,** 917–927
41. Wellen, K. E., Uysal, K. T., Wiesbrock, S., Yang, Q., Chen, H., and Hotamisligil, G. S. (2004) *Endocrinology* **145,** 2214–2220

0163-769X/05/$20.00/0
Printed in U.S.A.

Endocrine Reviews 26(3):439–451

doi: 10.1210/er.2005-0005

Adiponectin and Adiponectin Receptors

Takashi Kadowaki and Toshimasa Yamauchi

Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan; Department of Integrated Molecular Science on Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan; and Core Research for Evolutional Science and Technology of Japan Science and Technology Agency, Kawaguchi 332-0012, Japan

Metabolic syndrome is thought to result from obesity and obesity-linked insulin resistance. Obesity in adulthood is characterized by adipocyte hypertrophy. Adipose tissue participates in the regulation of energy homeostasis as an important endocrine organ that secretes a number of biologically active "adipokines."

Heterozygous peroxisome proliferator-activated receptor-γ knockout mice were protected from high-fat diet induced obesity, adipocyte hypertrophy, and insulin resistance. Systematic gene profiling analysis of these mice revealed that adiponectin/Acrp30 was overexpressed. Functional analyses including generation of adiponectin transgenic or knockout mice have revealed that adiponectin serves as an insulin-sensitizing adipokine. In fact, obesity-linked down-regulation of adiponectin was a mechanism whereby obesity could cause insulin resistance and diabetes.

Recently, we have cloned adiponectin receptors in the skeletal muscle (AdipoR1) and liver (AdipoR2), which appear to comprise a novel cell-surface receptor family. We showed that AdipoR1 and AdipoR2 serve as receptors for globular and full-length adiponectin and mediate increased AMP-activated protein kinase, peroxisome proliferator-activated receptor-α ligand activities, and glucose uptake and fatty-acid oxidation by adiponectin. Obesity decreased expression levels of AdipoR1/R2, thereby reducing adiponectin sensitivity, which finally leads to insulin resistance, the so-called "vicious cycle." Most recently, we showed that osmotin, which is a ligand for the yeast homolog of AdipoR (PHO36), activated AMPK via AdipoR in C2C12 myocytes. This may facilitate efficient development of adiponectin receptor agonists.

Adiponectin receptor agonists and adiponectin sensitizers should serve as versatile treatment strategies for obesity-linked diseases such as diabetes and metabolic syndrome. (*Endocrine Reviews* 26: 439–451, 2005)

I. Introduction
II. Identification and Molecular Structure
  A. Identification
  B. Molecular structure and multimeric form of adiponectin
III. Adiponectin and Insulin Resistance
  A. Low plasma adiponectin levels and insulin resistance
  B. Insulin-sensitizing effects of adiponectin
IV. Mouse Models
V. Mechanism of Action of Adiponectin
  A. Insulin-sensitizing actions
  B. Antiatherosclerotic actions
VI. Alterations in Adiponectin Gene Are Associated with Human Diabetes
VII. Cloning of Adiponectin Receptors AdipoR1 and AdipoR2
VIII. Regulation of Adiponectin Receptors
  A. Regulation of expression levels of AdipoR1 and AdipoR2
IX. Adiponectin Hypothesis
X. Adiponectin and Adiponectin Receptors as Therapeutic Targets
  A. Up-regulation of plasma adiponectin
  B. Up-regulation of adiponectin receptors and development of AdipoRs agonists
  C. Pleiotropic effects of adiponectin in relation to metabolic syndrome

Abbreviations: ACC, Acetyl-coenzyme-A carboxylase; AMPK, AMP-activated protein kinase; apoE, apolipoprotein E; EGF, epidermal growth factor; gAd, globular adiponectin; HF, high-fat; HMW, high molecular weight; PI, phosphatidylinositol; PPAR, peroxisome proliferator-activated receptor; PR, pathogenesis-related; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; TG, triglyceride.

Endocrine Reviews is published bimonthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

I. Introduction

HIGH-FAT (HF) diet-induced insulin resistance associated with obesity is a major risk factor for diabetes and cardiovascular diseases, the prevalence of which is increasing sharply (1, 2). However, the molecular basis for this association remains to be elucidated. The adipose tissue itself serves as the site of triglyceride (TG) storage and free fatty acid/glycerol release in response to changing energy demands (1). Adipose tissue also participates in the regulation of energy homeostasis as an important endocrine organ that secretes a number of biologically active adipokines such as free fatty acid (3), adipsin (4), leptin (5), plasminogen activator inhibitor-1 (6), resistin (7), and TNF-α (8). Adiponectin is one such adipokine that has recently attracted much attention. In this review, we will describe recent progress made in adiponectin research with particular emphasis on the role of adiponectin in the regulation of insulin sensitivity and the development of insulin resistance. Other aspects of adiponectin research have been reviewed elsewhere (9–14).

II. Identification and Molecular Structure

A. *Identification*

Adiponectin was originally identified independently by four groups using different approaches. Mouse cDNAs for

adiponectin, termed Acrp30 (15) and AdipoQ (16), were cloned by differential display before and after differentiation of mouse 3T3-L1 and 3T3-F442A cells. Human adiponectin cDNA was isolated by large-scale random sequencing of a 3′-directed human adipose tissue cDNA library (17). Human adiponectin was also purified from plasma as a gelatin binding protein, GBP28 (18).

B. Molecular structure and multimeric form of adiponectin

Adiponectin structurally belongs to the complement 1q family (19–21) (Fig. 1) and is known to form a characteristic homomultimer (22) (Fig. 2). It has been demonstrated that simple SDS-PAGE under nonreducing and non-heat-denaturing conditions clearly separates multimeric species of adiponectin (23) (Fig. 2). Adiponectin in human or mouse serum and adiponectin expressed in NIH-3T3 cells or *Escherichia coli* forms a wide range of multimers from trimers and hexamers to high molecular weight (HMW) multimers (23) such as dodecamers and 18 mers, as demonstrated by ourselves and other groups (22, 24, 25) (Fig. 2).

Adiponectin can exist as full-length or a smaller, globular fragment; however, almost all adiponectin appears to exist as full-length adiponectin in plasma. Lodish's group reported that a small amount of globular adiponectin was detected in human plasma (26) (Fig. 1). It has been proposed that the globular fragment is generated by proteolytic cleavage, and recently it has been shown that the cleavage of adiponectin by leukocyte elastase secreted from activated monocytes and/or neutrophils could be responsible for the generation of the globular fragment of adiponectin (27). However, the pathophysiological importance of adiponectin cleavage by leukocyte elastase *in vivo* remains to be determined.

Oligomer formation of adiponectin depends on disulfide bond formation mediated by Cys-39 (28). Interestingly, a mutant adiponectin with a substitution of Cys by Ser at codon 39, which formed a trimer and readily underwent proteolytic cleavage, showed much more potent bioactivity, such as reduction of glucose output from primary hepatocytes, than wild-type adiponectin with a HMW.

Hydroxylation and glycosylation of the four lysines in the collagenous domain of adiponectin have been shown to play important roles in enhancing the ability of subphysiological concentrations of insulin to inhibit gluconeogenesis in hepatocytes (29). Adiponectin was reported to be an α2,8-linked disialic acid-containing glycoprotein, although the biological functions of the disialic acid epitope of adiponectin remain to be elucidated (30).

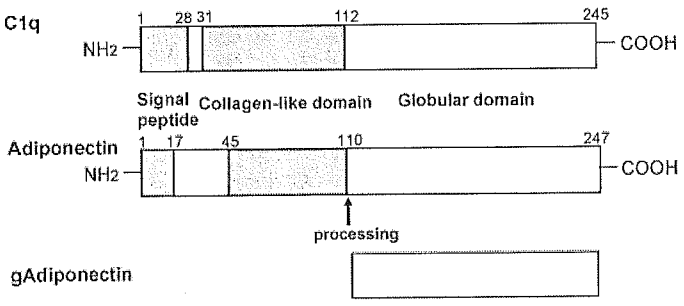

FIG. 1. Structure and domains of adiponectin. Adiponectin, also known as Acrp30, AdipoQ, and GBP28, was originally identified independently by four groups using different approaches (15–18). Adiponectin is composed of an N-terminal collagen-like sequence and a C-terminal globular region. A small amount of a processed globular form was reported to be present in human plasma (26).

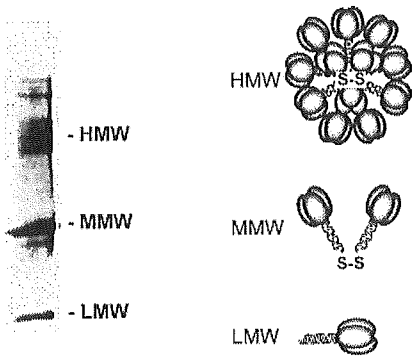

FIG. 2. Multimer formation of adiponectin. Human serum was subjected to SDS-PAGE under nonreducing, non-heat-denaturing conditions, and multimer forms of adiponectin were detected using antiadiponectin antibody (23). MMW, Medium molecular weight; LMW, low molecular weight; S–S, disulfide bridge.

III. Adiponectin and Insulin Resistance

A. Low plasma adiponectin levels and insulin resistance

Spiegelman's group reported that adiponectin expression is exclusive to adipose tissue and that the mRNA expression of adiponectin was reduced in obese diabetic murine model db/db mice (16). Plasma levels of adiponectin have also been reported to be significantly reduced in obese/diabetic mice and humans (16, 31, 32). Moreover, plasma adiponectin levels have been shown to be decreased in patients with cardiovascular diseases (33, 34), hypertension (35), or metabolic syndrome (36). Thus, reductions in plasma adiponectin levels are commonly observed in a variety of states frequently associated with insulin resistance. However, whether this apparent parallelism between low plasma adiponectin levels and insulin resistance represents a cause and effect relationship was not known.

B. Insulin-sensitizing effects of adiponectin

The insulin-sensitizing effect of adiponectin was first identified by three independent groups in 2001 (26, 31, 37). We previously generated heterozygous PPAR (peroxisome proliferator-activated receptor) γ knockout mice that remained insulin-sensitive on a HF diet (38). In an attempt to identify insulin-sensitizing molecules secreted from white adipose tissue of heterozygous PPARγ knockout mice, oligonucleotide microarray analysis was carried out using white adipose tissue (39). Adiponectin as well as leptin expression was up-regulated. Leptin was previously shown to be a major insulin-sensitizing adipokine (40).

To verify the direct insulin-sensitizing effect of adiponectin *in vivo*, an insulin-resistant lipoatrophic diabetic mouse model that displays both adiponectin and leptin deficiency was employed (41). Replenishment of a physiological dose of recombinant adiponectin to the lipoatrophic diabetic mice significantly ameliorated insulin resistance (31). Moreover,

insulin resistance in lipoatrophic mice was completely reversed by the combination of physiological doses of adiponectin and leptin, but only partially by either adiponectin or leptin alone (31). These data clearly indicate that adiponectin has a direct insulin-sensitizing action. These data also suggest that leptin and adiponectin may be the two major insulin-sensitizing hormones secreted from adipose tissue.

We also studied whether adiponectin can improve insulin resistance and diabetes in murine models of type 2 diabetes, characterized by obesity, insulin resistance, and hyperglycemia. Serum adiponectin levels were decreased in KKAy mice on a HF diet compared with those under a high-carbohydrate diet (31) (Fig. 3). Lower serum adiponectin levels in KKAy mice on the HF diet were partially restored by replenishment of recombinant adiponectin. Importantly, replenishment of adiponectin significantly ameliorated HF diet-induced insulin resistance and hypertriglyceridemia (31) (Fig. 3). These data suggest that the insulin resistance associated with HF diets and obesity is caused at least in part by the decreases in adiponectin linked to those circumstances. The data suggest that the fat-derived hormone adiponectin is decreased in obesity and deficient in lipoatrophy, and that reduction in adiponectin plays causal roles in the development of insulin resistance in these models.

Scherer's group has reported that an acute increase in circulating adiponectin levels triggers a transient decrease in basal glucose levels by inhibiting both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production in both wild-type mice and a type 2 diabetes mouse model (37, 42), consistent with the proposal that adiponectin sensitizes the body to insulin. Lodish's group reported that a proteolytic cleavage product of adiponectin increases fatty-acid oxidation in muscle and causes a decrease in plasma glucose and weight loss in mice (26).

These data raise the possibility that the replenishment of adiponectin may provide a novel treatment modality for insulin resistance and type 2 diabetes.

IV. Mouse Models

The chronic effects of adiponectin on insulin resistance *in vivo* were investigated by generating globular adiponectin transgenic mice (43, 44) or adiponectin-deficient mice (45–47). Globular adiponectin transgenic mice were generated and crossed with ob/ob mice (45). Globular adiponectin transgenic ob/ob mice showed partial amelioration of insulin resistance and diabetes, but not of obesity (43). These data suggested that chronic elevation of globular adiponectin has a direct insulin-sensitizing effect independent of white adipose tissue mass.

Scherer's group reported that transgenic mice with a deletion in the collagenous domain of adiponectin displayed 3-fold elevated levels of circulating adiponectin, raised lipid clearance and lipoprotein lipase activity, and improved insulin-mediated suppression of endogenous glucose production, thereby improving insulin sensitivity (44). In rats, sustained peripheral expression of adiponectin by the transgene also offset the development of diet-induced obesity (48).

Globular adiponectin transgenic mice were also crossed with apolipoprotein E (apoE)-deficient mice to study whether globular adiponectin can inhibit atherosclerosis *in vivo* (43). apoE-Deficient mice are hypercholesterolemic and spontaneously develop severe atherosclerosis. We compared the extent of atherosclerotic lesions of globular adiponectin transgenic apoE-deficient mice to that in control apoE-deficient mice. Although serum parameters such as total cholesterol, TG, glucose, and insulin were not altered, the *en face* Sudan IV-positive lesion areas of the arch and the descending aorta were significantly smaller in globular adiponectin transgenic apoE-deficient mice than in control apoE-deficient littermates (43). Similar results were obtained by using adenoviral-mediated overexpression of adiponectin in apoE knockout mice (49). Thus, overexpression of adiponectin resulted in marked reduction of atherosclerotic lesion formation. Together with the observations that adiponectin can ameliorate diabetes and hyperlipidemia, adiponectin can re-

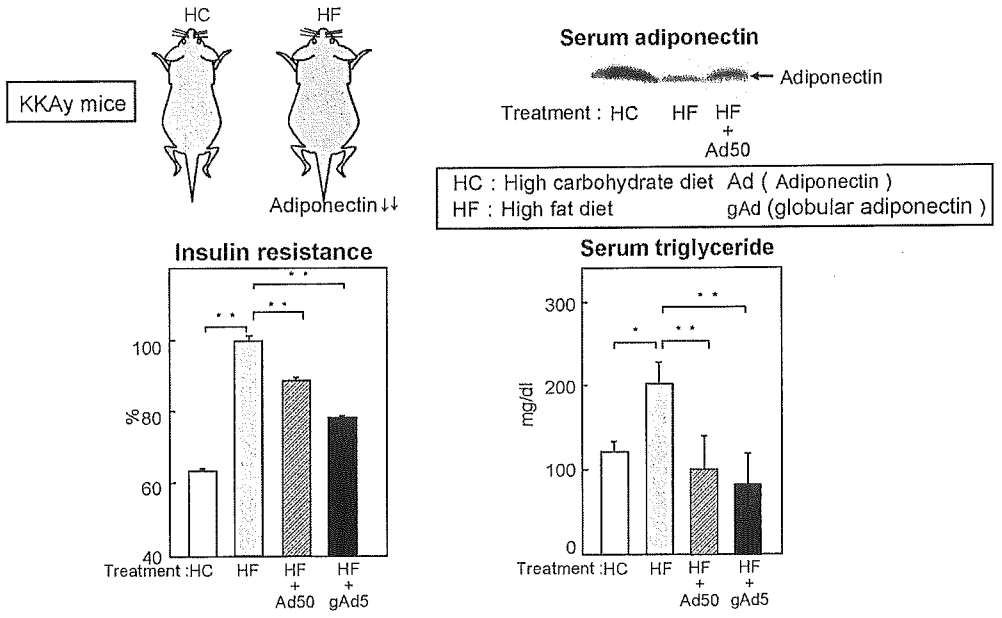

FIG. 3. Replenishment of adiponectin reversed insulin resistance and metabolic syndrome in a murine model of type 2 diabetes. Serum adiponectin levels were decreased in mice on a HF diet compared with those in mice on a high-carbohydrate (HC) diet. Lower serum adiponectin levels in mice on the HF diet were partially restored compared with those in mice on the HC diet by replenishment of recombinant adiponectin, which significantly ameliorated HF diet-induced insulin resistance. *, $P < 0.05$; **, $P < 0.01$, between the two groups indicated.

duce atherosclerosis both via direct effects on vascular wall and via reduction in risk factors.

To determine the physiological role of adiponectin, we and others have generated adiponectin knockout mice and reported that adiponectin-deficient mice exhibited characteristics of the metabolic syndrome such as insulin resistance, glucose intolerance, hyperlipidemia, and hypertension (35, 45, 46).

We and others also studied the role of adiponectin in vascular wall using adiponectin knockout mice (45, 50). We placed a cuff around the femoral artery to induce inflammation of the adventitia and subsequent neointimal formation 2 wk after cuff placement. Intimal thickness was significantly greater (2-fold) in adiponectin knockout mice than in the wild-type mice. Thus, adiponectin plays a protective role against neointimal formation in response to injury (45, 50).

V. Mechanism of Action of Adiponectin

A. *Insulin-sensitizing actions*

1. Adiponectin reduces tissue TG content and up-regulates insulin signaling. Interestingly, in skeletal muscle, adiponectin increased expression of molecules involved in fatty-acid transport such as CD36, in combustion of fatty-acid such as acyl-coenzyme A oxidase, and in energy dissipation such as uncoupling protein 2. These changes led to decreased tissue TG content in skeletal muscle (31).

Increased tissue TG content has been reported to interfere with insulin-stimulated phosphatidylinositol (PI) 3-kinase activation and subsequent glucose transporter 4 translocation and glucose uptake, leading to insulin resistance (3). Thus, decreased tissue TG content in muscle may contribute to improved insulin signal transduction. This was demonstrated in skeletal muscle of lipoatrophic mice treated with adiponectin, in which increases in insulin-induced tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 and insulin-stimulated phosphorylation of Akt were seen (31).

2. Adiponectin activates PPARα. Based on the data that treatment of lipoatrophic or obese diabetic mice with adiponectin or overexpression of adiponectin in ob/ob mice resulted in increased expression levels of PPARα target genes such as CD36, acyl-coenzyme A oxidase, and uncoupling protein 2, we hypothesized that adiponectin could activate PPARα (31) (Fig. 4).

Consistent with this hypothesis, adiponectin indeed increased the expression levels of PPARα *in vivo* (31). These data suggested that adiponectin increased fatty-acid combustion and energy consumption, presumably via PPARα activation at least in part, which led to decreased TG content in the liver and skeletal muscle and thus coordinately increased *in vivo* insulin sensitivity.

Endogenous PPARα ligand activities were measured *in vitro* to further clarify the mechanisms by which adiponectin activated PPARα (31, 43). Interestingly, the treatment of C2C12 myocytes with adiponectin for 6 h significantly increased PPARα ligand activities (43) and at the same time fatty-acid oxidation *in vitro*.

3. Adiponectin activates AMP kinase. We next examined the effects of treatment of adiponectin for a shorter time period

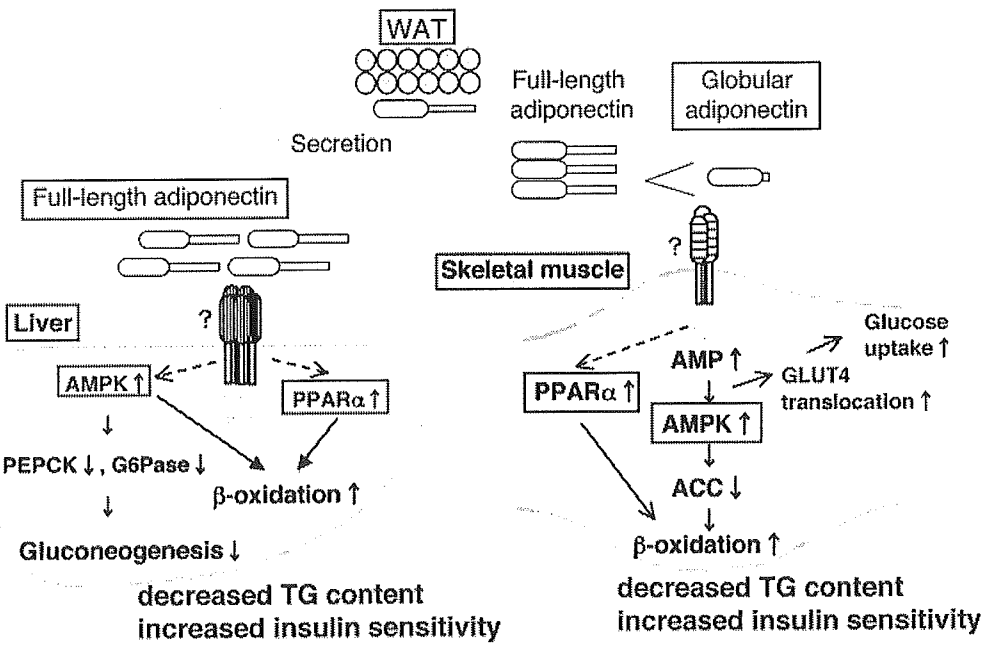

FIG. 4. Adiponectin can activate AMPK and PPARα in the liver and skeletal muscle. In skeletal muscle, both globular and full-length adiponectin activate AMPK, thereby stimulating phosphorylation of ACC, fatty-acid oxidation, and glucose uptake. Adiponectin activates PPARα, thereby also stimulating fatty-acid oxidation and decreasing tissue TG content in muscle. In the liver, only full-length adiponectin activates AMPK, thereby reducing molecules involved in gluconeogenesis and increasing phosphorylation of ACC and fatty-acid oxidation. Adiponectin activates PPARα, thereby stimulating fatty-acid oxidation and decreasing tissue TG content in the liver. These alterations all increase insulin sensitivity *in vivo* (101). WAT, White adipose tissue; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase G6Pase.

(51). Treatment of C2C12 myocytes with adiponectin for 1 h stimulated fatty-acid oxidation. Although actinomycin D had no effect on the increase in fatty-acid oxidation stimulated by adiponectin for 1 h, it suppressed fatty-acid oxidation stimulated by the PPARα agonist Wy-14,643. Moreover, treatment of C2C12 myocytes for 1 h stimulated glucose uptake. We hypothesized that adiponectin may stimulate β-oxidation and glucose uptake via AMP-activated protein kinase (AMPK) during a period shorter than 6 h (51).

Globular adiponectin and full-length adiponectin stimulated phosphorylation and activation of AMPK in skeletal muscle, whereas only full-length adiponectin did so in the liver (51). In parallel with its activation of AMPK, adiponectin stimulated phosphorylation of acetyl coenzyme-A carboxylase (ACC), fatty-acid combustion, glucose uptake, and lactate production in myocytes, and also stimulated phosphorylation of ACC and caused a reduction in molecules involved in gluconeogenesis in the liver, which can account for the acute glucose-lowering effects of adiponectin *in vivo* (51). Blocking AMPK activation by use of a dominant negative mutant inhibited each of these effects, indicating that stimulation of glucose utilization and fatty-acid combustion by adiponectin occurs through activation of AMPK. Our data may provide a novel paradigm that an adipocyte-derived hormone activates AMPK, thereby directly regulating glucose metabolism and insulin sensitivity *in vitro* and *in vivo* (51) (Fig. 4).

The group of Lodish and Ruderman also showed that the adiponectin/ACRP30 globular domain enhanced muscle fat oxidation and glucose transport via AMPK activation and ACC inhibition (52). More recently, AMPK was reported to be involved in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes (53). Because leptin has also been shown to stimulate AMPK in skeletal muscle (54), activation of AMPK may be a common mechanism by which insulin-sensitizing adipokines such as adiponectin and leptin increase insulin sensitivity.

Scherer's group also reported that in adiponectin transgenic mice, reduced expression of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase was associated with elevated phosphorylation of AMPK in liver (44). The same group reported that adiponectin is found as two forms in serum, as a lower molecular weight trimer-dimer and a HMW complex (28). Female subjects display significantly higher levels of the HMW complex in serum than do male subjects (23, 28, 55–57). Levels of the HMW complex appeared to be negatively regulated by insulin. In accordance with this, the amount of HMW adiponectin complex, but not the total amount of adiponectin, was recently reported to be correlated with a thiazolidinedione-mediated improvement in insulin sensitivity (55).

B. Antiatherosclerotic actions

Adiponectin has been reported to have direct antiatherosclerotic effects (58–67). Adiponectin was demonstrated to strongly inhibit the expression of adhesion molecules, including intracellular adhesion molecule-1, vascular cellular adhesion molecule-1, and E-selectin (Fig. 5). Adiponectin was also shown to inhibit TNF-α-induced nuclear factor-κB activation through the inhibition of IκB phosphorylation (61). Suppression of nuclear factor-κB by adiponectin might be a major molecular mechanism for the inhibition of monocyte adhesion to endothelial cells (62). Adiponectin also inhibits the expression of the scavenger receptor class A-1 of macrophages, resulting in markedly decreased uptake of oxidized low-density lipoprotein by macrophages and inhibition of foam cell formation (63). In addition, in cultured smooth muscle cells, adiponectin attenuated DNA synthesis

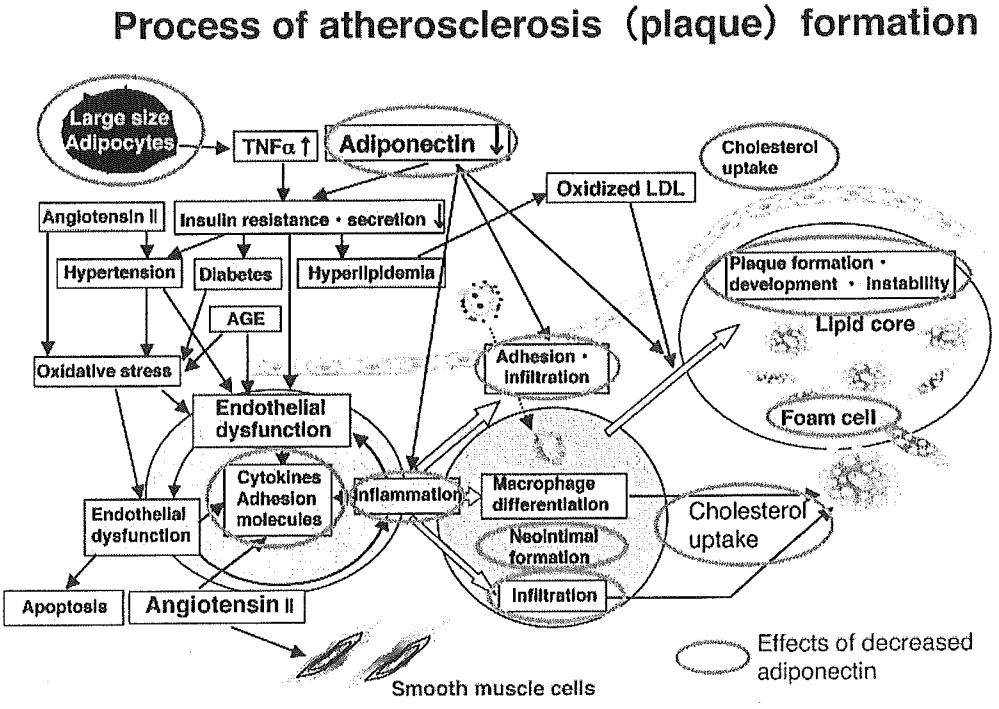

FIG. 5. Suppression of atherosclerosis by adiponectin. Adiponectin inhibits atherosclerosis and plaque formation at least via suppression of two processes: 1) suppression of neointimal formation by inhibiting the expressions of inflammatory cytokines and adhesion molecules; and 2) suppression of uptake of cholesterol by inhibiting the expression of scavenger receptors. LDL, Low-density lipoprotein.

induced by growth factors including platelet-derived growth factor, heparin-binding epidermal growth factor (EGF)-like growth factor, basic fibroblast growth factor, and EGF, as well as cell proliferation and migration induced by heparin-binding EGF-like growth factor (64). This inhibition was shown to be attributable to the inhibition of signal transduction through ERK. More recently, selective suppression of endothelial cell apoptosis via AMPK activation by the HMW form of adiponectin has been reported (65).

VI. Alterations in Adiponectin Gene Are Associated with Human Diabetes

Independent of these functional analyses carried out *in vitro* and in animal models, data from human genetic studies on adiponectin also support the role of adiponectin as a determinant of susceptibility to insulin resistance and type 2 diabetes. By use of affected sib-pair analysis, complete genome mapping of type 2 diabetes genes in Japanese was performed (68). The genome scans revealed at least nine chromosomal regions linked to type 2 diabetes in Japanese people. Among these, three chromosomal regions (3q, 15q, and 20q) are the same regions as previously reported in other ethnic groups. Among these three chromosomal regions, interestingly, the 3q27 chromosomal region contains the adiponectin gene.

We screened for the adiponectin gene and identified 10 relatively common single nucleotide polymorphisms (SNPs) in the Japanese population (Fig. 6). One such SNP, SNP 276 in intron 2 (G *vs.* T), showed interesting phenotypes with respect to plasma adiponectin levels, insulin resistance, and susceptibility to type 2 diabetes (69) (Fig. 7). Subjects with the G/G genotype had lower plasma adiponectin levels than those with the T/T genotype. Subjects with the G/G genotype at position 276 had a higher insulin resistance index than those with T/T. Importantly, subjects with the G/G genotype at position 276 were at increased risk for type 2 diabetes. The odds ratio was slightly greater than 2 (69) (Fig. 8). Similar associations for the adiponectin gene with susceptibility to type 2 diabetes have also been reported in other ethnic groups (70–72). In German and American Caucasians, the SNP 276, either independently or as a haplotype together with SNP 45 in exon 2, was shown to be associated with obesity and insulin resistance (71, 72). In French Caucasians, two SNPs in the promoter region of the adiponectin gene, SNP-11377 and SNP-11391, were significantly associated with hypoadiponectinemia and type 2 diabetes (70). Taken together, these data strongly support the hypothesis that adiponectin plays a pivotal role in the pathogenesis of type 2 diabetes.

Several cross-sectional studies have reported that adiponectin levels were decreased in subjects with type 2 diabetes and are inversely correlated with insulin resistance. However, no studies had investigated whether adiponectin protects subjects from diabetes or the extent of risk of developing diabetes in subjects with hypoadiponectinemia. Recently, matched case-control studies in subjects recruited from a large cohort have examined the protective effect of adiponectin against diabetes. One study was performed in severely obese Pima Indian subjects, who have the highest known prevalence of obesity and type 2 diabetes in the world, to assess the role of adiponectin independent of the effects of obesity (73). Subjects with high concentrations of adiponectin were 40% less likely to develop type 2 diabetes than those with low concentrations after adjustment for body mass index (BMI), indicating that adiponectin could be used as a predictor of future development of type 2 diabetes in addition to the established risk parameters, such as BMI.

In addition to the relatively common SNPs, eight mutations in the human adiponectin gene have been reported (69, 74, 75), some of which were significantly related to diabetes and hypoadiponectinemia (23, 75). Among human adiponectin mutations, Arg112Cys and Ile164Thr mutants did not assemble into trimers, which caused impaired secretion from the cell (23). These mutants are clinically associated with hypoadiponectinemia. The Gly84Arg and Gly90Ser mutants were able to assemble into trimers and hexamers but were unable to form HMW multimers (the HMW multimers are thought to be larger than heximers), which are clinically associated with diabetes. These data raised the possibility that HMW multimers have more potent insulin-sensitizing effects than trimers and hexamers (23).

These data suggest that impaired multimerization of adiponectin may be among the causes of a diabetic phenotype or hypoadiponectinemia in subjects having these mutations. Thus, not only the total concentrations but also the multimer distribution should always be considered when interpreting plasma adiponectin levels in health as well as various disease states (23–25).

VII. Cloning of Adiponectin Receptors AdipoR1 and AdipoR2

We believe that cloning of the adiponectin receptor should facilitate studies on the regulation of glucose and lipid me-

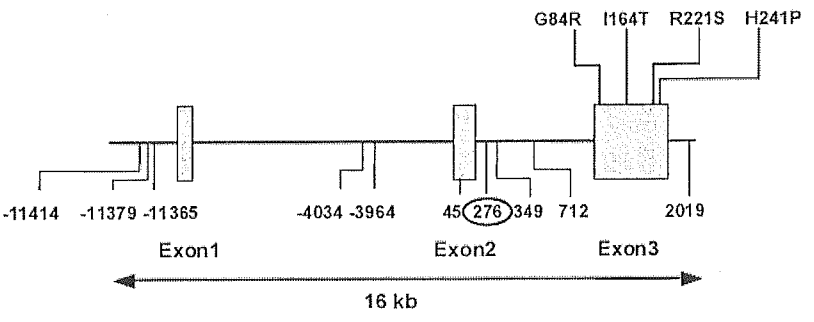

FIG. 6. Schema of genomic structure and polymorphic variants of the adiponectin gene. Exon-intron organization of the gene is indicated by *closed boxes*. *Arrows* show the positions of the polymorphic variants identified. *Numbers* indicate locations relative to the A of the ATG of the initiator Met of the adiponectin gene. Rare mutations with amino acid substitutions are also described (23, 70, 75).

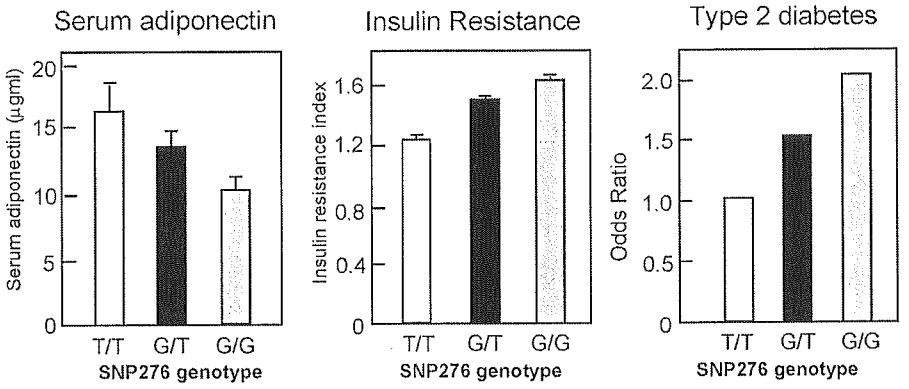

FIG. 7. Effects of SNP 276 in intron 2 of the adiponectin gene on serum adiponectin, insulin resistance, and susceptibility to type 2 diabetes. The effects of SNP 276 in intron 2 on plasma adiponectin levels, insulin resistance, and susceptibility to type 2 diabetes were studied. On the *left*, subjects with the G/G genotype had lower plasma adiponectin levels than those with the T/T genotype. In the *middle*, subjects with the G/G genotype at position 276 had a higher insulin resistance index than those with T/T. Importantly, on the *right*, subjects with the G/G genotype at position 276 were at increased risk for type 2 diabetes. The odds ratio was slightly greater than 2.

tabolism, the molecular causes of diabetes and atherosclerosis, and the development of antidiabetic and antiatherosclerotic drugs. We isolated cDNA for adiponectin receptors (AdipoR) that mediate the antidiabetic effects from human skeletal muscle cDNA library by screening for globular adiponectin binding (76).

The cDNA analyzed encoded a protein designated human AdipoR1 (Fig. 8) (76). This protein is conserved from yeast to man (especially in the seven transmembrane domains). Interestingly, this yeast homolog YOL002c plays a key role in metabolic pathways that regulate lipid metabolism such as fatty-acid oxidation (77).

Because there may be two distinct adiponectin receptors, we searched for a homologous gene in the human and mouse databases. We found only one gene that was significantly homologous (67% identity in amino acids) to AdipoR1, which was termed AdipoR2 (Fig. 8) (76). AdipoR1 was ubiquitously expressed and most abundantly expressed in skeletal muscle, whereas AdipoR2 was most abundantly expressed in mouse liver. It was reported that adiponectin receptors were expressed in pancreatic β-cells, and that fatty acids may regulate their expression levels (78). GH is reported to be a positive regulator of AdipoR2 in 3T3-L1 adipocytes (79).

AdipoR1 and AdipoR2 appeared to be integral membrane proteins; the N terminus was internal, and the C terminus was external, which is opposite to the topology of all other reported G protein-coupled receptors (Fig. 8) (76). AdipoR1 and AdipoR2 may form both homo- and heteromultimers. Scatchard plot analysis revealed that AdipoR1 is a receptor for globular adiponectin, whereas AdipoR2 is a receptor for full-length adiponectin (76). Suppression of AdipoR1 with small interfering RNA (siRNA) reduced the increase in fatty-acid oxidation by globular adiponectin. Suppression of AdipoR2 with siRNA reduced the increase in fatty-acid oxidation by full-length adiponectin (Fig. 9) (76).

Thus, we have isolated cDNA-encoding adiponectin receptors (AdipoR1 and R2). Expression of AdipoR1/R2 or suppression of AdipoR1/R2 supports our conclusion that AdipoR1 and R2 serve as receptors for globular and full-length adiponectin and mediate increased AMPK, PPARα ligand activities and fatty-acid oxidation and glucose uptake by adiponectin (Fig. 9) (57).

Lodish's group reported that T-cadherin was capable of binding adiponectin in C2C12 myoblasts, but not in the liver or hepatocytes (80).

VIII. Regulation of Adiponectin Receptors

A. Regulation of expression levels of AdipoR1 and AdipoR2

We first examined whether the expressions of AdipoR1 and/or AdipoR2 were regulated under physiological and/or pathophysiological states (81). The levels of AdipoR1 and AdipoR2 mRNA expression in the liver and skeletal muscle increased after fasting, and refeeding rapidly restored these to levels equal to the original fed state. AdipoR1 and AdipoR2 mRNA increased significantly in skeletal muscle of

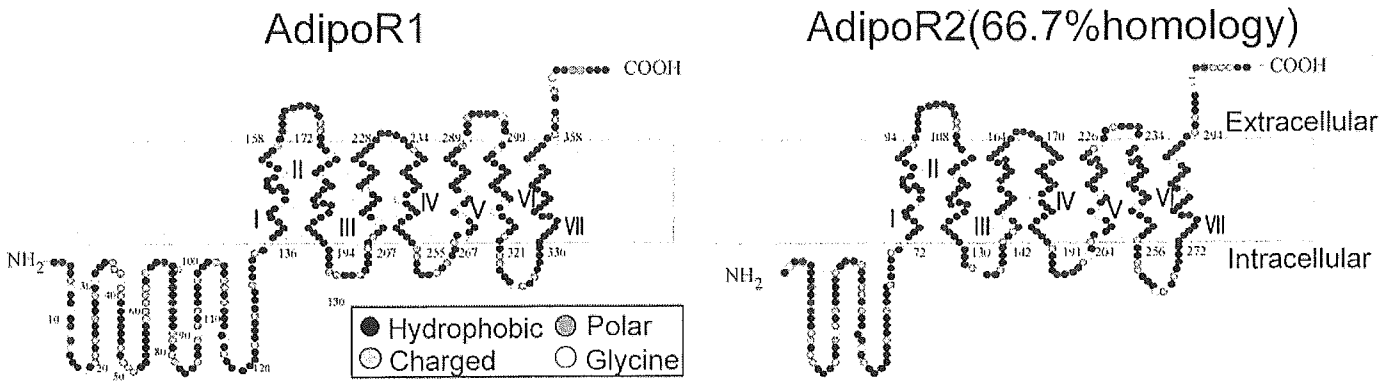

FIG. 8. Proposed structure of adiponectin receptors and their expression in various tissues. cDNA encoding adiponectin receptors (AdipoR1 and R2) were isolated. AdipoR1 was ubiquitously expressed and most abundantly expressed in skeletal muscle, whereas AdipoR2 was most abundantly expressed in mouse liver (76).

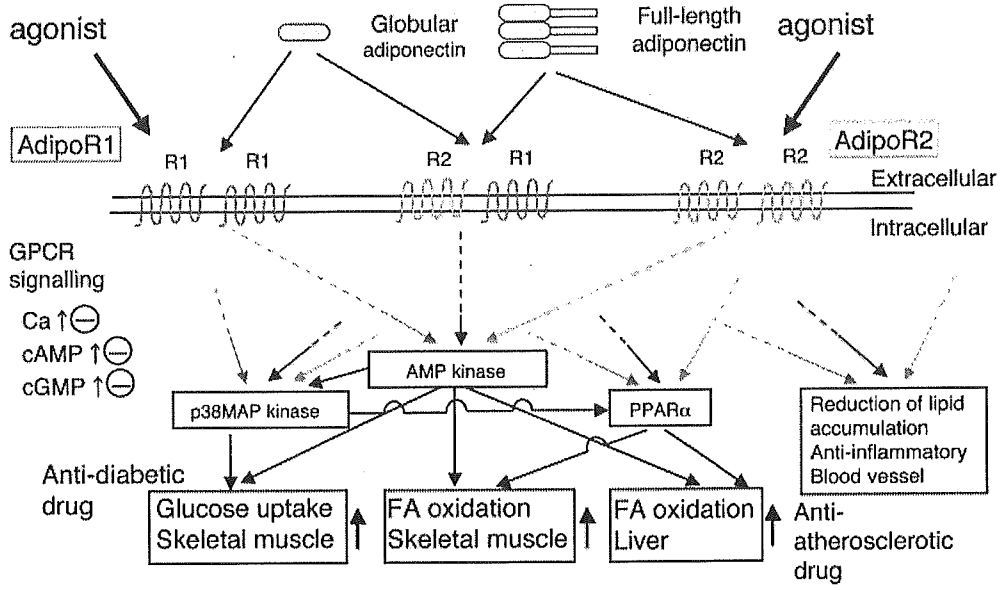

FIG. 9. Molecular mechanisms of adiponectin action. cDNA encoding adiponectin receptors (AdipoR1 and R2) was isolated. Expression of AdipoR1/R2 or suppression of AdipoR1/R2 supports the conclusion that AdipoR1 and R2 serve as receptors for globular and full-length adiponectin and mediate increased AMPK, PPARα ligand activities, and fatty-acid oxidation and glucose uptake by adiponectin. Molecular cloning of AdipoR1 and R2 should facilitate the designing of novel antidiabetic and antiatherogenic drugs with AdipoR1 and R2 as molecular targets (76, 101). FA, Fatty acid; GPCR, G protein-coupled receptor.

mice rendered hypoinsulinemic/hyperglycemic with streptozotocin, and both AdipoR1 and AdipoR2 mRNA were almost completely restored by insulin treatment. These observations suggested that insulin may negatively regulate AdipoR1/R2 mRNA levels (81). The PI3-kinase inhibitor LY 294002 and constitutively active form of Foxo (Forkhead box, class O) 1 revealed that insulin repressed AdipoR1/R2 mRNA expressions via activation of PI3-kinase and inactivation of Foxo1 (81).

The expressions of both AdipoR1 and AdipoR2 were significantly decreased in muscle and adipose tissue of insulin-resistant ob/ob mice, which exhibited hyperglycemia and hyperinsulinemia, as compared with control mice (81) (Fig. 10). Scatchard plot analysis revealed that both high-affinity and low-affinity binding sites for globular adiponectin (gAd) and adiponectin binding in skeletal muscles of ob/ob mice were reduced as compared with those of wild-type mice, findings that are consistent with the fact that the numbers of both AdipoR1 and AdipoR2 were reduced. Moreover, adiponectin-induced activation of AMPK was impaired in skeletal muscle of ob/ob mice. These data suggest that adiponectin resistance was observed in ob/ob mice, which exhibited decreased expression levels of AdipoR1 and AdipoR2 (81) (Fig. 10).

We and others have previously shown that plasma adiponectin levels were decreased in obesity. This reduction may play a causal role in the development of insulin resistance. In the same study, we have also shown that obesity decreased the expression levels of AdipoR1/R2, thereby reducing adiponectin sensitivity, which finally leads to insulin resistance, the so-called "vicious cycle" (61) (Fig. 10).

A correlation has been reported between adiponectin receptor gene expression and insulin sensitivity in nondiabetic Mexican Americans with or without a family history of type 2 diabetes (82). Adiponectin receptor expression in skeletal muscle of type 2 diabetic patients was also reported to be decreased (83).

Our data suggest that not only agonism of AdipoR1/R2 but also strategies to increase AdipoR1/R2 may be a logical approach with which to provide a novel treatment modality for insulin resistance and type 2 diabetes.

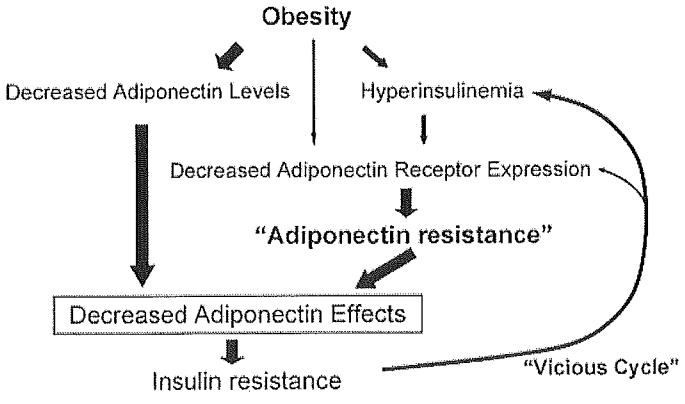

FIG. 10. Obesity, adiponectin resistance, and insulin resistance. Plasma adiponectin levels were decreased in obesity, which may play causal roles in the development of insulin resistance. The expression levels of AdipoR1/R2 were also decreased in obesity. Obesity decreased expression levels of AdipoR1/R2, thereby reducing adiponectin sensitivity, which finally led to insulin resistance, the so-called "vicious cycle". These data also suggest that not only agonism of AdipoR1/R2 but also strategies to increase AdipoR1/R2 may be a logical approach to provide a novel treatment modality for insulin resistance and type 2 diabetes (81).

IX. Adiponectin Hypothesis

Based upon the significant body of evidence discussed in this review, obtained from our and other laboratories, we propose the following adiponectin hypothesis (Fig. 11). Reduced adiponectin levels were caused by interactions of genetic factors such as SNPs in the adiponectin gene itself and environmental factors causing obesity such as a HF diet. Reduced adiponectin actions also resulted from down-regulation of adiponectin receptors linked to obesity. These reductions of adiponectin actions may play a crucial causal role in the development of insulin resistance, type 2 diabetes, metabolic syndrome, and atherosclerosis (Fig. 11).