

# PPAR $\gamma$ Ligands Increase Expression and Plasma Concentrations of Adiponectin, an Adipose-Derived Protein

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Insulin resistance and its dreaded consequence, type 2 diabetes, are major causes of atherosclerosis. Adiponectin is an adipose-specific plasma protein that possesses anti-atherogenic properties, such as the suppression of adhesion molecule expression in vascular endothelial cells and cytokine production from macrophages. Plasma adiponectin concentrations are decreased in obese and type 2 diabetic subjects with insulin resistance. A regimen that normalizes or increases the plasma adiponectin might prevent atherosclerosis in patients with insulin resistance. In this study, we demonstrate the inducing effects of thiazolidinediones (TZDs), which are synthetic PPAR $\gamma$  ligands, on the expression and secretion of adiponectin in humans and rodents *in vivo* and *in vitro*. The administration of TZDs significantly increased the plasma adiponectin concentrations in insulin resistant humans and rodents without affecting their body weight. Adiponectin mRNA expression was normalized or increased by TZDs in the adipose tissues of obese mice. In cultured 3T3-L1 adipocytes, TZD derivatives enhanced the mRNA expression and secretion of adiponectin in a dose- and time-dependent manner. Furthermore, these effects were mediated through the activation of the promoter by the TZDs. On the other hand, TNF- $\alpha$ , which is produced more in an insulin-resistant condition, dose-dependently reduced the expression of adiponectin in adipocytes by suppressing its promoter activity. TZDs restored this inhibitory effect by TNF- $\alpha$ . TZDs might prevent atherosclerotic vascular disease in insulin-resistant patients by inducing the production of adiponectin through direct effect on its promoter and antagonizing the effect of TNF- $\alpha$  on the adiponectin promoter. *Diabetes* 50:2094–2099, 2001

Insulin resistance is characterized by a reduced sensitivity of insulin to target tissues. The insulin-resistant state is a common basis for hypertension and coronary artery disease, as well as type 2 diabetes (1). Although obesity is a common cause of insulin resistance, the molecular link between increased adiposity and human diseases remains unclear (2). Adipose tissue synthesizes and secretes biologically active molecules that might be called “adipocytokines” (3–5). Dysregulation of adipocytokines in obesity might play a role in the development of insulin resistance and vascular disorders. A cytokine, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), is overproduced by adipose tissue in obese mice and causes systemic insulin resistance by interfering with the insulin-signaling cascade (6,7). Leptin, a central regulator of adiposity, also affects glucose homeostasis (8,9). More recently, resistin, which is a novel adipose-specific cysteine-rich protein, has been found to impair insulin sensitivity and glucose tolerance (10). TNF- $\alpha$  enhances the cell surface expression of adhesion molecules on vascular endothelial cells (11). Thus, TNF- $\alpha$  may also contribute to the initial step of atherosclerosis. Furthermore, increased expression and secretion of plasminogen activator inhibitor 1 (PAI-1) and angiotensinogen in the adipose tissue may link obesity to thrombotic vascular disease and hypertension (12,13).

Adiponectin is a product of the *apM1* gene, which is specifically and highly expressed in human adipose tissue (14). The protein belongs to the soluble defense collagen superfamily and is present in human plasma at a concentration of 5–30  $\mu\text{g/ml}$  (15). This protein, also referred to as gelatin-binding protein 28, was independently purified from plasma using gelatin affinity chromatography (16). In mechanically injured vascular walls, adiponectin could be detected in the subendothelial space (17). A mouse homologue was identified in two independent laboratories and designated AdipoQ and ACRP30, respectively (18,19). The expression of AdipoQ and ACRP30 was limited to the adipose tissue and reduced in obesity. Plasma adiponectin concentrations and mRNA expression were decreased in obese humans, despite increased adiposity, and in patients with type 2 diabetes with insulin resistance (15,20,21).

Hyperinsulinemic-euglycemic clamp studies have revealed that the degree of hypo adiponectinemia is closely related

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DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PAI-1, plasminogen activator inhibitor 1; PGZ, pioglitazone; PPAR, peroxisome proliferator-activated receptor; RGZ, rosiglitazone; TGZ, troglitazone; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TZD, thiazolidinedione.

to the degree of insulin resistance (22,23). In vitro, adiponectin attenuates the TNF- $\alpha$ -induced expression of adhesion molecules in vascular endothelial cells and the secretion of TNF- $\alpha$  from monocyte macrophages (24–26). These results suggest that adiponectin may work as an anti-atherogenic factor. In fact, marked decreases in the plasma adiponectin concentrations were observed in patients with coronary artery disease and macroangiopathy related to diabetes (21,24). Therefore, the high incidence of coronary artery disease in patients with insulin resistance may be partially attributed to hypo adiponectinemia, and a regimen that could increase the plasma adiponectin might prevent the development of atherosclerosis in those with insulin resistance.

The peroxisome proliferator-activated receptor (PPAR)- $\gamma$  is the master regulator of adipocyte differentiation and controls many adipocyte genes. Its synthetic ligands, thiazolidinediones (TZDs), are a new class of antidiabetic drugs that improve insulin action. In the current study, we show that TZDs can markedly enhance the expression and secretion of adiponectin in vitro and in vivo through the activation of its promoter, and that these compounds also antagonize the suppressive effect of TNF- $\alpha$  on the production of adiponectin. Through the induction of adiponectin, TZDs might prevent atherosclerosis in diabetic patients with insulin resistance.

## RESEARCH DESIGN AND METHODS

### Effect of troglitazone on plasma adiponectin concentration in humans.

A total of 29 subjects (16 men, 13 women, average age  $50 \pm 3$  years, BMI  $29.1 \pm 0.8$  kg/m<sup>2</sup>) with glucose intolerance were eligible for the study and gave written informed consent. The study was performed in accordance with good clinical practice and with the approval of the respective institutional review boards and the ethical committee of Osaka University Graduate School of Medicine. The subjects were randomly allocated to either placebo or troglitazone (TGZ) 100 or 200 mg twice daily for 12 weeks. Fasting plasma glucose, immunoreactive insulin, HbA<sub>1c</sub>, and adiponectin concentrations were measured before and after treatment. The plasma adiponectin concentrations were measured by sandwich enzyme-linked immunosorbent assay as previously described (15).

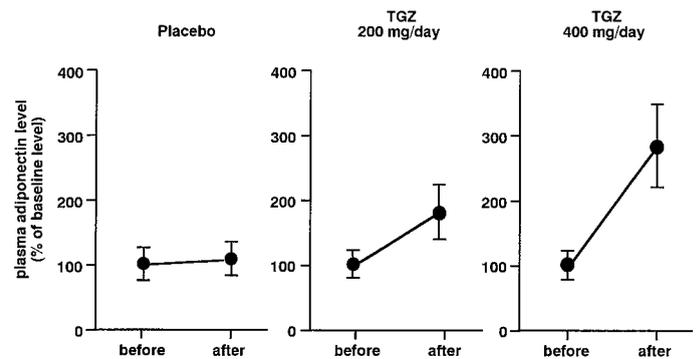
**Effect of TZDs on adipose expression and plasma level of adiponectin/ACRP30 in mice.** Eight-week old male C57BL/KsJ (*db+/+*) ( $n = 20$ ) and C57BL/KsJ (*db/db+*) ( $n = 20$ ) mice (Clea Japan, Tokyo) were fed powder chow (CRF-1; Oriental Kobo, Suita, Japan). The chow consisted of 53.5% (wt/wt) carbohydrate, 5.9% (wt/wt) fat, 23.1% (wt/wt) protein, and 3.3% (wt/wt) dietary fiber. At 13 weeks, the mice were divided into four groups. Each group, consisting of five *db+/+* and five *db/db+* mice, was fed powder chow containing either 0.2% TGZ, 0.01% pioglitazone (PGZ), 0.5% rosiglitazone (RGZ), or no TZDs (control group), respectively. The mice were killed 2 weeks later after an overnight fast.

Total RNA extracted from subcutaneous adipose tissues was electrophoresed and transferred to nylon membranes (24). The membranes were hybridized with murine adiponectin homologue/ACRP30 or a TNF- $\alpha$  cDNA probe labeled with [ $\alpha$ -<sup>32</sup>P]dCTP.

Plasma adiponectin concentrations were measured by Western blotting. An equal aliquot of plasma was resolved on a 12.5% SDS-PAGE gel, followed by electrophoretic transfer to a nitrocellulose membrane. The signal was detected using the ECL system (Amersham) with a rabbit polyclonal antibody against murine adiponectin/ACRP30 and anti-rabbit HRP antibody.

**Effect of TZDs and TNF- $\alpha$  on the expression and secretion of adiponectin/ACRP30 in 3T3-L1 adipocytes.** Mouse 3T3-L1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) and differentiated with DMEM supplemented with 5  $\mu$ g/ml of insulin, 0.5 mmol/l 1-methyl-3-isobutyl-xanthin, and 1  $\mu$ mol/l dexamethazone 2 days after reaching confluence. On day 7, the indicated concentrations of TZDs and/or murine TNF- $\alpha$  (Pepro Tech EC) were added to the media at the indicated concentrations for 24 h.

Adiponectin/ACRP30 mRNA levels were measured by Northern blotting. An aliquot of the media from 12 to 24 h after stimulation was subjected to



**FIG. 1.** Effect of TGZ on plasma adiponectin level in humans. Effects of TGZ on human plasma adiponectin levels were studied in 29 mildly overweight subjects with glucose intolerance. The subjects were divided randomly into three groups: placebo group ( $n = 8$ ), TGZ 200 mg/day group ( $n = 10$ ), and TGZ 400 mg/day group ( $n = 11$ ). Each group was treated with the indicated amount of TGZ for 12 weeks. Data are represented as percentage change relative to the mean levels before the treatment. Values are means  $\pm$  SE.

Western blotting to detect the amount of adiponectin/ACRP30 that was secreted.

**Effect of TZDs and TNF- $\alpha$  on the promoter activity of adiponectin in 3T3-L1 adipocytes.** On day 7 after differentiation, the media of the 3T3-L1 cells in 12-well plates were changed to OPTI-MEM (Life Technologies), and the cells were transfected with plasmid using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's instructions. Transfections were performed using 10 ng of pRL (Renilla luciferase)-SV40 (internal standard) along with pGL3-basic plasmid containing the adiponectin promoter or with pGL3-basic alone. Three hours later, an equal amount of DMEM or DMEM containing TZDs or TNF- $\alpha$  was added to the media. At 48 h after transfection, luciferase reporter assays were performed using the Dual-Luciferase Reporter Assay System (Promega), and the transfection efficiencies were normalized to the Renilla luciferase activity.

**Statistical analysis.** The data are expressed as means  $\pm$  SE. Differences were analyzed by the paired Student's *t* test or one-way analysis of variance.  $P < 0.05$  was considered statistically significant.

## RESULTS

**Elevation of plasma adiponectin level by TZD in humans.** We first tested the effect of insulin-sensitizing TZD on the plasma concentrations of adiponectin in humans. TGZ was the only available TZD when this study was performed. The study subjects were middle-aged, mildly overweight, and had impaired glucose tolerance. The TGZ-treated and placebo groups were well matched for BMI, body fat composition, and fasting plasma glucose. No subjects had any subjective side effects or persistent abnormalities in their laboratory variables. The administration of TGZ for 12 weeks did not change the BMIs significantly. As shown in Fig. 1, the subjects in the placebo group did not show any significant change in their plasma adiponectin concentration ( $2.6 \pm 0.2$  vs.  $2.7 \pm 0.2$   $\mu$ g/ml). However, the administration of 200 mg of TGZ per day for 12 weeks significantly increased the plasma adiponectin concentration ( $3.3 \pm 0.6$  vs.  $5.9 \pm 1.4$   $\mu$ g/ml,  $P < 0.01$ ). Furthermore, a marked increase in the plasma adiponectin was detected in the subjects treated with TGZ 400 mg/day ( $3.0 \pm 0.3$  vs.  $8.8 \pm 2.0$   $\mu$ g/ml,  $P < 0.001$ ).

**Augmentation of adipose expression and plasma concentration of adiponectin by TZDs in mice.** To further elucidate the effect of TZDs on adiponectin in vivo, we treated lean and obese (*db/db*) mice with various TZDs for 2 weeks and measured the plasma adiponectin and the mRNA levels in white adipose tissue (Fig. 2A and B). In the chow-treated control group, the plasma concentration and

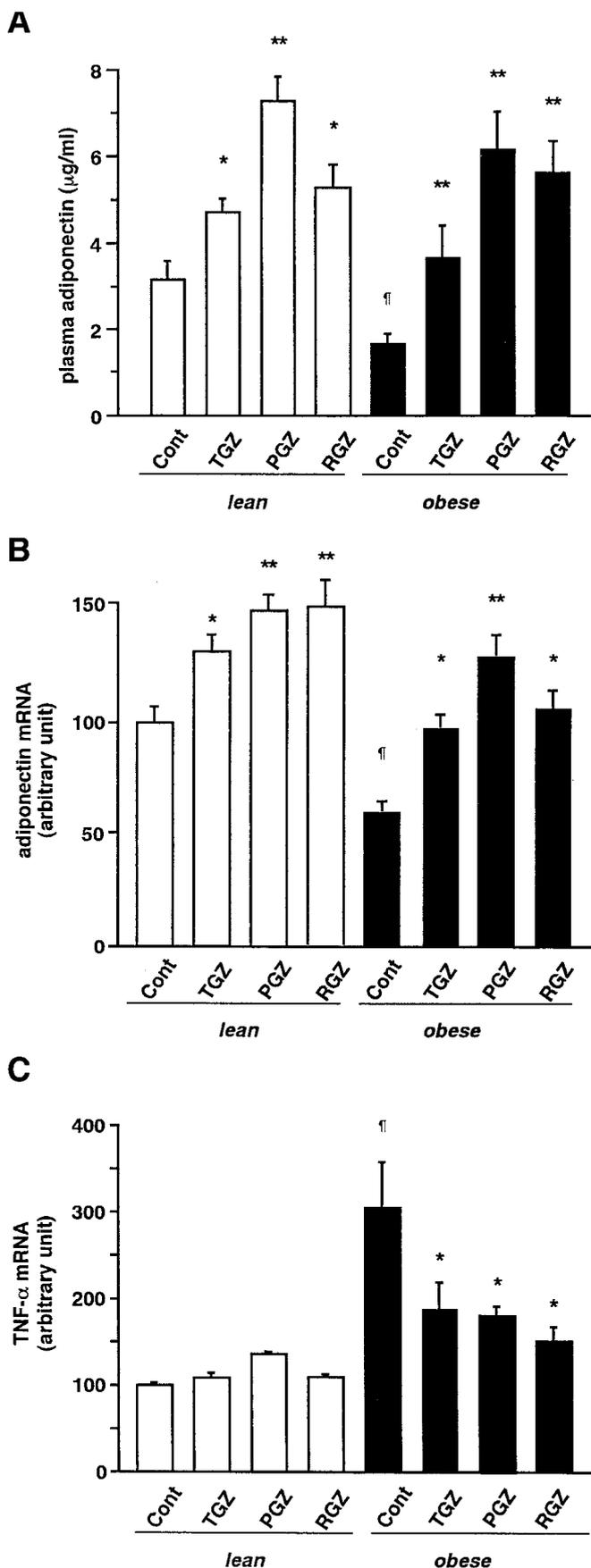


FIG. 2. Effect of TZDs on plasma levels and adipose expression of adiponectin in mice. Effect of TZDs on plasma concentrations (A), mRNA levels of adiponectin (B), or TNF- $\alpha$  (C) in lean and obese mice were investigated. Male 13-week-old lean and *db/db* mice were divided

into four groups: control, TGZ, PGZ, and RGZ groups. After 2 weeks of the treatment of 0.2% (wt/wt) TGZ, 0.01% PGZ, or 0.5% RGZ, plasma and subcutaneous fat RNA samples were prepared and analyzed. Values are means  $\pm$  SE. \* $P$  < 0.05, \*\* $P$  < 0.01 compared with control chow-treated lean or obese group, respectively.  $\ddagger P$  < 0.05 compared with lean control group.

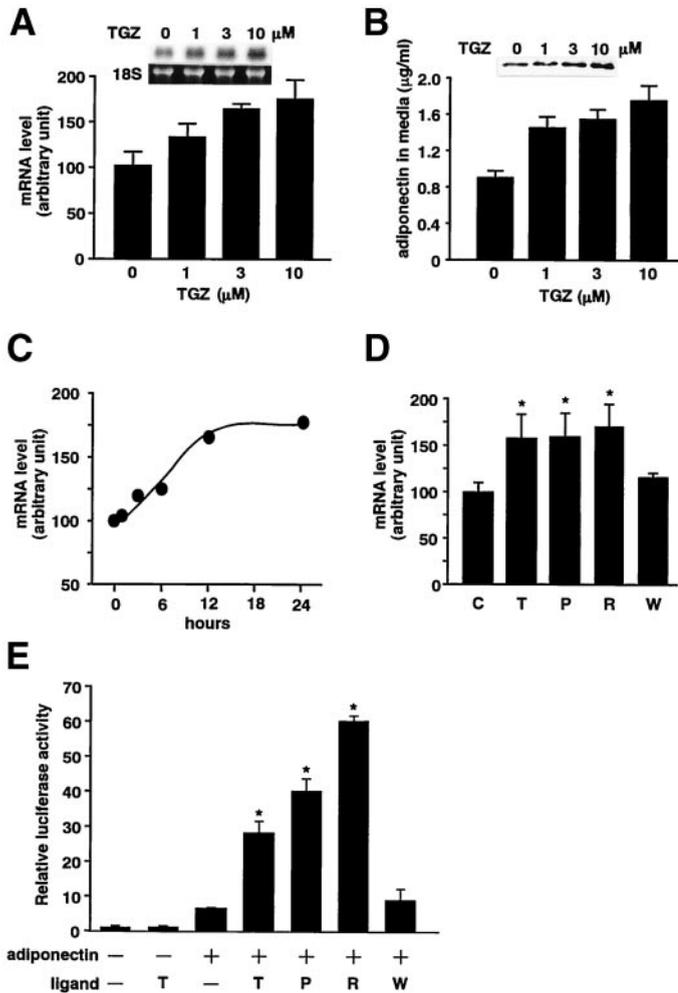
mRNA levels of adiponectin were significantly lower than in the obese mice, as previously described (18). Treatment with TGZ, PGZ, or RGZ increased the plasma adiponectin and mRNA levels in both lean and obese mice. These treatments caused the reduced plasma adiponectin and mRNA levels to return to normal in obese mice and to normal or higher than normal levels in the chow-treated lean mice. Because ectopic expression of adiponectin mRNA was not observed in other tissues with these treatments (such as the liver and kidney), the increased plasma adiponectin caused by TZDs is most likely attributable to increased production in adipose tissues (data not shown). TNF- $\alpha$  was shown to increase with obesity and was considered as one of the key molecules for insulin resistance (6,7). TNF- $\alpha$  mRNA levels were elevated in the adipose tissue of obese mice, and the elevated TNF- $\alpha$  mRNA was decreased in the adipose tissue by the treatment of these compounds, consistent with previous reports (7,27) (Fig. 2C).

**Inducible effects of TZDs on the expression and secretion of adiponectin in 3T3-L1 adipocytes.** We investigated the effect of TZDs on the regulation of adiponectin gene expression in 3T3-L1 adipocytes. Incubation with TGZ enhanced adiponectin mRNA expression and adiponectin secretion into the media in a dose-dependent fashion (Fig. 3A and B). The induction of adiponectin mRNA expression by TGZ was detected after 3 h and reached a maximum after 12 h of treatment (Fig. 3C). Two other TZDs (PGZ and RGZ) also had a similar inducing effect on adiponectin mRNA expression, although Wy14643, a synthetic PPAR $\alpha$  ligand, had no significant effect (Fig. 3D). We next studied the effects of PPAR $\gamma$  activators on the -2.0-kb human adiponectin promoter activity (28). TGZ, PGZ, and RGZ enhanced the adiponectin promoter activity by 4.7-, 6.6-, and 10.0-fold, respectively, although Wy14,643 had no effect (Fig. 3E). These results suggest that the *in vivo* induction of adiponectin expression by TZDs is most likely secondary to the direct activation of the adiponectin promoter.

**Suppressive effects of TNF $\alpha$  on the expression and secretion of adiponectin in 3T3-L1 adipocytes.** TNF- $\alpha$  has been shown to increase in obesity and is considered one of the key molecules involved in insulin resistance (6,7). TNF- $\alpha$  dose-dependently reduced the expression and the secretion of adiponectin in 3T3-L1 adipocytes (Fig. 4A and B). The reducing effect of TNF- $\alpha$  on adiponectin mRNA was antagonized by coinubation with TGZ (Fig. 4C). This result was confirmed in an analysis of adiponectin promoter activity. Incubation with 0.1 ng/ml TNF- $\alpha$  decreased promoter activity by 80%. When these cells were coinubated with TGZ, the promoter activity returned to basal level (Fig. 4D).

## DISCUSSION

Adiponectin, an adipocyte-derived factor, possesses anti-atherogenic properties and is decreased in patients with

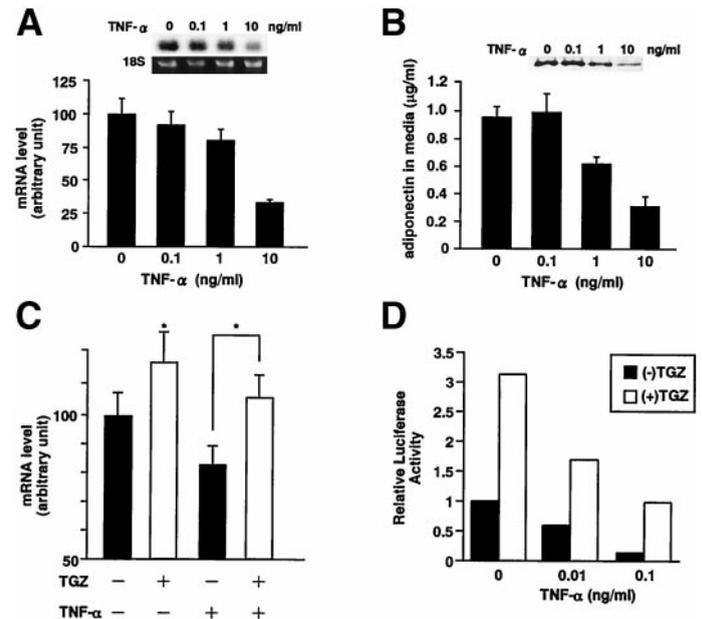


**FIG. 3.** Effects of TZDs on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose effect of TGZ on the adiponectin mRNA level (A) and the secreted amount in media (B) is shown. After differentiation-induction on day 7, 3T3-L1 cells were treated with the indicated concentrations of TGZ, 0, 1, 3, and 10  $\mu\text{mol/l}$  for 24 h. Five micrograms of total RNA was subjected to Northern blotting and quantified and normalized relative to the 18-s rRNA signal. All mRNAs are plotted as percentage change relative to mRNA level by 0  $\mu\text{mol/l}$  TGZ treatment. The amount of adiponectin secreted into media was measured by quantitative Western blotting. The inset shows a representative picture of Northern and Western blotting that was quantified. Time curve of the effect of 10  $\mu\text{mol/l}$  TGZ on adiponectin mRNA (C) is shown. After differentiation-induction on day 7, 3T3-L1 cells were treated with 10  $\mu\text{mol/l}$  TGZ for the indicated time. Northern blotting and quantification was performed as described in the legend of A. After differentiation-induction on day 7, 3T3-L1 cells were treated with 10  $\mu\text{mol/l}$  TGZ, PGZ, RGZ, and Wy14643, respectively (D). Northern blotting and quantification was performed as described for A. A 2.0-kb adiponectin promoter was subcloned into pGL3-luciferase vector (E). Adiponectin-luciferase and pRL-SV40 vector were cotransfected into day 7–differentiated 3T3-L1 cells. For each 12-well dish, 1  $\mu\text{g}$  adiponectin-luciferase and 10 ng pRL-SV40 were used. Three hours after transfection, the cells were switched to medium containing vehicle (DMSO), or 10  $\mu\text{mol/l}$  TGZ (T), PGZ (P), RGZ (R), or Wy14643 (W) for 24 h. Firefly and Renilla luciferase reporter activity was measured as described in RESEARCH DESIGN AND METHODS. Adiponectin-luciferase (firefly) activities were normalized by pRL-SV40 Renilla luciferase activity. Values are means  $\pm$  SE from three independent experiments. \* $P < 0.01$  compared with control values.

insulin resistance (15,21–26). In the current study, we demonstrated that TZD derivatives increased the plasma adiponectin concentrations in humans and rodents through the activation of its promoter.

During the early phase of atherosclerosis, circulating monocytes attach to injured endothelial cells through

adhesion molecules and invade the subintimal space (23,29). The monocytes transform into macrophages and secrete various cytokines and growth factors that promote smooth muscle cell proliferation. Adiponectin inhibits the expression of adhesion molecules and prevents the attachment of monocytes in TNF- $\alpha$ -stimulated human aortic endothelial cells (24,25). This protein also dramatically suppresses the secretion of TNF- $\alpha$  from macrophages and foam cell formation (26,30). These data suggest that adiponectin works as an anti-atherogenic molecule. Although its receptor has not been identified, adiponectin modulates NF $\kappa$ B signaling, at least partly, through a cAMP-dependent pathway (25). The plasma adiponectin concentration is decreased in insulin-resistant states, such as obesity and type 2 diabetes (15,21). Recently, Kissebah et al. (31) demonstrated two quantitative trait loci that influence the phenotypes of the insulin resistance–metabolic syndrome. One is located on chromosome 3q27, where the adiponectin gene is encoded (28). In light of these data, hypoadiponectinemia may play a role in the development of atherosclerotic vascular disease in patients with insulin



**FIG. 4.** Effect of TNF- $\alpha$  on expression and secretion of adiponectin in 3T3-L1 adipocytes. Dose effect of TNF- $\alpha$  on the adiponectin mRNA level (A) and the secreted amount in media (B) are shown. After differentiation-induction on day 7, 3T3-L1 cells were treated with indicated concentrations of TNF- $\alpha$  for 24 h. One nanogram per milliliter of TNF- $\alpha$  corresponds to 57 pmol/l. Total RNAs (5  $\mu\text{g}$ ) were subjected to Northern blotting and quantified as described in the legend of Fig. 3A. The amount of adiponectin secreted into media was measured by quantitative Western blotting. Inset shows a representative picture of Northern and Western blotting that was quantified. After differentiation-induction on day 7, 3T3-L1 cells were treated with or without 1 ng/ml TNF- $\alpha$  and 10  $\mu\text{mol/l}$  TGZ for 24 h (C). Total RNAs (5  $\mu\text{g}$ ) were subjected to Northern blotting and quantified as described in the legend of Fig. 3A. Effects of TNF- $\alpha$  on the adiponectin promoter activity were analyzed as described in Fig. 3E (D). Adiponectin-luciferase and pRL-SV40 vector were cotransfected into day 7–differentiated 3T3-L1 cells. For each 12-well dish, 1  $\mu\text{g}$  adiponectin-luciferase and 10 ng pRL-SV40 were used. Three hours after transfection, an equal amount of DMSO or DMEM containing TZDs or TNF- $\alpha$  was supplemented to medium. At 48 h after transfection, cells were harvested, and firefly and Renilla luciferase reporter activity was measured as described in RESEARCH DESIGN AND METHODS. Adiponectin-luciferase (firefly) activity was normalized by pRL-SV40 Renilla luciferase activity. Values are means  $\pm$  SE from three independent experiments. \* $P < 0.01$  compared with control values.

resistance. The mechanisms that control the plasma adiponectin concentration have not been elucidated.

Many adipose-specific genes are regulated by adiposity. Previously, we have shown that weight reduction therapy increased the plasma adiponectin concentration by 40–60% (21). In this report, TZD derivatives enhanced the expression of adiponectin mRNA in adipose cells and dramatically increased the plasma adiponectin concentration. TZDs are specific ligands for PPAR $\gamma$ , which is a key transcriptional factor that induces adipocyte differentiation by activating the expression of adipocyte-specific genes and which is also known as an insulin sensitizer in vivo, presumably by increasing the number of mature adipocytes that can respond to an enhancing effect of insulin on glucose disposal (32–35). In many clinical trials, TZDs were shown to have a preventive effect against arteriosclerosis, although the net effect of PPAR $\gamma$  activation on atherosclerosis remains controversial (33,34,36–39). Increased adiponectin expression and increased plasma adiponectin concentrations might explain the anti-atherogenic effect of these compounds. A 2.0-kb fragment of the human adiponectin promoter had adipose-specific promoter activity and *cis*-elements for several adipogenic transcriptional factors, including CCAAT enhancer binding protein and sterol regulatory element binding protein (28,40). The promoter activity of adiponectin was markedly enhanced by the PPAR $\gamma$  ligands, TZDs, although we were unable to identify a putative PPAR $\gamma$ -responsive element in this region in the same case of leptin (41). Thus, TZDs might activate the adiponectin promoter by a pathway other than its direct action on PPAR $\gamma$ . However, we observed that cotransfection of a dominant-negative form of PPAR $\gamma$  diminished the action of TZDs on adiponectin promoter (data not shown). TZDs therefore may enhance adiponectin promoter activity through an unidentified element responsive to PPAR $\gamma$ . Detailed promoter analyses need to be performed.

The mechanism responsible for the decreased adiponectin concentration in insulin resistance has been obscure. TNF- $\alpha$  is one of the candidate molecules responsible for causing insulin resistance (6,7). Here we demonstrated that the expression and secretion of adiponectin from adipocytes were significantly reduced by TNF- $\alpha$  in a dose- and time-dependent manner via its promoter activity. The expression of adiponectin mRNA was reduced in the adipose tissue of insulin-resistant obese humans and rodents, where TNF- $\alpha$  production was increased (18,20). Therefore, increased TNF- $\alpha$  might be partially responsible for the decreased adiponectin production in obesity. The current study also showed that the suppressive effect of TNF- $\alpha$  on adiponectin could be antagonized by TZDs in tissue culture through its direct action on the adiponectin promoter. Hence, the ability of TZDs to enhance adiponectin mRNA expression might be attributable to both the direct activation of the promoter and an inhibitory effect on the TNF- $\alpha$ -mediated suppression of adiponectin mRNA expression. This was reflected in the in vivo study, where TZDs normalized or even increased the adiponectin levels in both adipose tissue and the plasma in insulin-resistant obese animals and normalized the elevated adipose TNF- $\alpha$  mRNA levels. Conversely, we demonstrated in our previous studies that adiponectin inhibited the TNF- $\alpha$  signaling

pathway in endothelial cells and reduced TNF- $\alpha$  production in macrophages (24–26,30). Crystal structure analysis revealed topologic homology among the mouse adiponectin homologue, ACRP30, and TNF- $\alpha$ , which have an evolutionary link (42). Taking all of this into account, a tempting hypothesis is that adiponectin and TNF- $\alpha$  may antagonize each other or perform opposite functions locally in adipose tissue and/or remotely in the arterial wall. This hypothesis is reinforced by the recent work by Fruebis et al. (43), who found that the administration of the globular domain of ACRP30 increased free fatty acid oxidation and decreased plasma glucose in mice, reflecting an improvement in insulin resistance. Further studies of in vivo models with overexpressed or disrupted adiponectin gene will be necessary to clarify the role of this molecule on the pathophysiology of insulin resistance.

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