Complex Distribution, Not Absolute Amount of Adiponectin, Correlates with Thiazolidinedione-mediated Improvement in Insulin Sensitivity* 

Received for publication, October 9, 2003, and in revised form, December 23, 2003 
Published, JBC Papers in Press, December 29, 2003, DOI 10.1074/jbc.M311113200

Utpal B. Pajvani‡, Meredith Hawkins§, Terry P. Combs‡, Michael W. Rajala*, Tom Doebber¶, Joel P. Berger†, John A. Wagner†, Margaret Wu†, Annemie Knopps‡, Anny H. Xiang**, Kristina M. Utzschneider***, Steven E. Kahn††, Jerrold M. Olefsky‡‡, Thomas A. Buchanan‡‡, and Philipp E. Scherer§§

From the ‡Department of Cell Biology and the §Diabetes Research and Training Center and Department of Medicine, Division of Endocrinology, Albert Einstein College of Medicine, Bronx, New York 10461, ¶Metabolic Disorders, Merck Research Laboratories, Rahway, New Jersey 07065, **SGS Biopharma, Antwerp, Belgium 12060, the Departments of ***Preventive Medicine and ¶¶Medicine, University of Southern California Keck School of Medicine, Los Angeles, California 90033, the §§Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, Veterans Affairs Puget Sound Health Care System and University of Washington, Seattle, Washington 98108; and the §§Department of Endocrinology and Metabolism, University of California San Diego, La Jolla, California 92093

Adiponectin is an adipocyte-specific secretory protein that circulates in serum as a hexamer of relatively low molecular weight (LMW) and a larger multimeric structure of high molecular weight (HMW). Serum levels of the protein correlate with systemic insulin sensitivity. The full-length protein affects hepatic glucoseogenesis through improved insulin sensitivity, and a proteolytic fragment of adiponectin stimulates β oxidation in muscle. Here, we show that the ratio, and not the absolute amounts, between these two oligomeric forms (HMW to LMW) is critical in determining insulin sensitivity. We define a new index, $S_A$, that can be calculated as the ratio of HMW/(HMW + LMW); $S_A$ improves with wild type littermates, as do type II diabetic patients compared with insulin-sensitive individuals. Furthermore, $S_A$ improves with peroxisome proliferator-activated receptor-γ agonist treatment (thiazolidinedione; TZD) in mice and humans. We demonstrate that changes in $S_A$ in a number of type 2 diabetic cohorts serve as a quantitative indicator of improvements in insulin sensitivity obtained during TZD treatment, whereas changes in total adiponectin levels do not correlate well at the individual level. Acute alterations in $S_A$ ($\Delta S_A$) are strongly correlated with improvements in hepatic insulin sensitivity and are less relevant as an indicator of improved muscle insulin sensitivity in response to TZD treatment, further underscoring the conclusions from previous clamp studies that suggested that the liver is the primary site of action for the full-length protein. These observations suggest that the HMW adiponectin complex is the active form of this protein, which we directly demonstrate in vivo by its ability to depress serum glucose levels in a dose-dependent manner.

Adipocytes, beyond their role in lipid storage, can influence whole-body metabolism through modulation of systemic free fatty acid levels as well as secretion of cell-specific proteins collectively known as adipokines. Recent work has underscored the importance of these adipocyte-secreted molecules in energy homeostasis and metabolism (1–3). Some of these adipokines have synergistic effects, whereas others, such as resistin and adiponectin/Acrp30 (adipocyte complement-related protein of 30 kDa), have competing effects; pharmacological doses of resistin deactivate the repressive effects of insulin on gluconeogenesis (4), whereas adiponectin increases insulin sensitivity, leading to enhanced inhibition of hepatic glucose output (5). Genetic variability within the adiponectin gene leads to alteration of serum levels of the protein and predisposes carriers of specific polymorphisms to insulin resistance (reviewed in Ref. 6). Decreased serum adiponectin is now considered a feature of obesity, and levels correlate with lowered indices of insulin sensitivity (7, 8), leading to the hypothesis that decreased serum adiponectin levels are contributory and not simply diagnostic of systemic insulin resistance.

The potential importance of adiponectin as a therapeutic target is underscored by the dramatic up-regulation of this adipokine in response to treatment with the antidiabetic, insulin-sensitizing agents known as thiazolidinediones (TZDs) (9–11). TZDs have proven to be active in animal models of genetic or acquired insulin resistance as well as in type 2 diabetic patients in clinical settings, suggesting that these drugs improve insulin sensitivity regardless of the underlying cause (12). However, the molecular mechanisms of TZD action are still not fully understood. The presumptive target of TZDs, peroxisome proliferator-activated receptor-γ (PPARγ), is predominantly expressed in adipose tissue, suggesting that adipocytes may play a critical role in mediating the antidiabetic effects of these drugs (13). This hypothesis has been supported by studies of “fatless” mice that displayed reduced metabolic improvement in response to TZD treatment (14). Subsequent studies in this same mouse model demonstrated amelioration of defects in insulin-stimulated glucose uptake in muscle with TZD administration but exacerbation of hepatic insulin resist-

* This work was supported by National Institutes of Health (NIH) Medical Scientist Training Grant T32-GM07288 (to U. B. P.), Medical Research Service Grants DK-62654 and DK-17047 (to S. E. K.), NIH Grant 2R01-DK46374 (to T. A. B.), and a research grant from Pfizer (to T. A. B.).
‡ To whom correspondence should be addressed: Dept. of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-2928; Fax: 718-430-8574; E-mail: scherer@aecom.yu.edu.

¶ The abbreviations used are: TZD, thiazolidinedione; LMW, low molecular weight form; HMW, high molecular weight form; PPARγ; peroxisome proliferator-activated receptor-γ; BMI, body mass index.
ance in these animals, leading to no significant net antidiabetic effect (15).

A number of studies have demonstrated that in mice and humans, TZD treatment effects transcriptional up-regulation accompanied by increased production and secretion of adiponectin from adipocytes (9–11). However, whether the TZD-mediated induction of adiponectin is causative or simply diagnostically improved insulin sensitivity remains under debate, since discordance between improvements in insulin sensitivity and induction in serum adiponectin levels has been reported (10). Although the vast majority of patients induce adiponectin expression and secretion in response to TZD treatment, only 50–70% of patients demonstrate clinically improved insulin sensitivity (reviewed in Ref. 16). This suggests that induction of adiponectin in any particular individual is neither predictive nor correlative to quantitative improvements in insulin sensitivity and led us to hypothesize that increased adiponectin levels alone are insufficient to explain the wide range of clinical responses to TZD treatment. We have recently demonstrated that adiponectin exists in minimally two forms in serum, as a hexamers referred to as a low molecular weight (LMW) complex and as a high molecular weight (HMW) complex consisting of 12–18 subunits (17). Here, we show that the ratio between these two oligomeric forms (HMW to LMW), not absolute amounts of circulating adiponectin, is critical in determining TZD-mediated improvements in insulin sensitivity.

MATERIALS AND METHODS

Velocity Sedimentation/Gel Filtration Chromatography for Separation of Adiponectin Complexes

5–20% sucrose gradients in 10 mM HEPES, pH 8, 125 mM NaCl were poured stepwise in 2-ml thin walled ultracentrifuge tubes (Becton Dickenson) and allowed to equilibrate overnight at 4 °C. Following layering of the sample on top (diluted 1:10 with 10 mM HEPES, pH 8, 125 mM NaCl), gradients were spun at 55,000 rpm for 4 h at 4 °C in a TLS55 rotor in a Beckman TL-100 tabletop ultracentrifuge. 150-μl gradient fractions were sequentially retrieved and analyzed by quantitative Western blot analysis as described below. Adiponectin complex distribution was independently confirmed by gel filtration chromatography, as previously described (17).

Immunoblotting

Separation of proteins by SDS-PAGE and immunoblotting were performed as described previously (18). Primary and secondary antibodies were diluted in TBS with 0.05% Tween 20 (TBS-T) and 1% bovine serum albumin. Horseradish peroxidase-conjugated secondary antibodies were detected with enhanced chemiluminescence according to the manufacturer’s instructions (Pierce). For quantitative Western blotting, proteins were transferred to BASi nitrocellulose (Schleicher & Schuell), and membranes were blocked with 5% nonfat dry milk (in TBS-T). An affinity-purified rabbit anti-adiponectin antibody raised against a peptide comprising the N-terminal hypervariable region (DVTITTEELAPALV, mouse; DQETTTQGPGV, human) was used; these antibodies recognize a single band of 30 kDa by Western analysis and have equal affinity for all oligomeric forms of the protein. Blots were probed with antibodies recognizing a single band of 30 kDa by Western analysis and have equal affinity for all oligomeric forms of the protein. Blots were probed with anti-phospho-Akt (Ser473) antibody (Cell Signaling Technology), anti-phospho-ERK (Thr202/Tyr204) antibody (Cell Signaling Technology), and anti-β-actin antibody (Sigma) as loading controls.

Results

In Vivo Animal Studies

Male db/db mice and control mice (db/+) (Jackson Laboratories) were housed at 5 mice/cage and allowed ad libitum access to ground Purina rodent chow 5001 and water. The animals were dosed daily by gavage with vehicle (0.25% carboxymethyl cellulose) or with or without 10 mg/kg per day of rosiglitazone for 11 days or 10 mg/kg per day of PARP1 antagonist for 7 days (compound 10, an O-arylmethane acid derivative (trithromethyl benzoxazole) (19)). Plasma adiponectin, glucose, insulin, and triglyceride levels were determined from blood obtained by tail bleeds at 3–4-day intervals during the studies. Wild type animals (C57Bl/6J) used in adipose extraction and adiponectin knockout animals for in vivo adiponectin activity studies were maintained in the same manner. All animal protocols were approved by the Albert Einstein Animal Committee.

Diabetic Mice Display Decreased HMW/Total Adiponectin Ratio Despite Comparable Levels of Total Serum Adiponectin—

Study A—This was a single center, double-blind, randomized, placebo-controlled, parallel group study with treatments including placebo and rosiglitazone (4 mg twice daily) for 14 days. Twenty non-diabetic mice were treated in this analysis (10/group). Plasma adiponectin concentration determination was obtained predose on day 1 (base line) and 2 h after the last dose on day 14. These subjects refrained from all other medication use from 14 days prior to completion of the trial and demonstrated no evidence or family history of diabetes mellitus. All subjects gave written informed consent, and the clinical protocol was approved by the Institutional Review Board.

Study B—The study group consisted of 18 middle-aged type 2 diabetic patients treated with either diet or sulfonylureas, the latter being withdrawn for 4 weeks prior to entry into the treatment phase of the study. The treatment phase consisted of daily administration of troglitazone at a dose of 200–800 mg/day for 12 weeks as previously reported (20). The data reported here are from the 15 individuals who underwent a frequently sampled intravenous glucose tolerance test to measure insulin sensitivity (S3, calculated by minimal model analysis) before and after troglitazone treatment. The protocol was approved by the human subjects review committee of the University of Washington, and informed consent was obtained from all subjects.

Study C—Hispanic women with a history of gestational diabetes mellitus were recruited to the Troglitazone in Prevention of Diabetes study if they had a sum of 5 glucose values on a 75-g oral glucose tolerance test that was above the median for a large cohort of women with gestational diabetes mellitus. Subjects underwent a frequently sampled intravenous glucose tolerance test to measure insulin sensitivity (S3, calculated by minimal model analysis) prior to enrollment and then again following 3 months of troglitazone treatment (400 mg/day). 108 women had measurements of insulin sensitivity at base line and three months post-treatment; plasma was obtained after overnight fasting from women who demonstrated >80% drug compliance (as measured by pill counts) and who had changes in insulin sensitivity between base line and 3 months that was either the lowest (n = 20) or the highest (n = 20) in the cohort. At base line, the groups had similar oral glucose tolerance test glucose sum (736 ± 15 versus 765 ± 15 mg/dl), S3 in women defined as low responders went from 3.3 ± 0.4 × 10−4 at base line to 3.8 ± 0.4 × 10−4 min−1 per microunit/ml after 3 months of troglitazone treatment. In the high responders, S3 went from 2.7 ± 0.3 × 10−4 at base line to 5.1 ± 0.7 × 10−4 min−1 per microunit/ml at 3 months. All subjects gave consent for participation in the study, which was approved by the institutional review board of the University of Southern California.

Study D—Ten subjects with type 2 diabetes and 17 non-diabetic control subjects (8 lean (BMI < 27 kg/m2) and 9 obese (BMI ≥ 27 kg/m2)) participated in the study. Subjects were excluded if they had active cardiac, hepatic, or renal disease or if they had long term complications from diabetes. The diabetic subjects were asked to discontinue taking their medications at least 2 weeks before their base-line studies. Each subject underwent a hyperinsulinemic-euglycemic glucose clamp at base line and again after 3 months of treatment with 600 mg/day troglitazone. Following a 10-h overnight fast, a catheter was inserted in an antecubital vein, and a constant infusion was started of [3-3H]glucose (0.25 μCi/min) (PerkinElmer Life Sciences). At 0700 h, another catheter was inserted in a retrograde fashion in a hand vein, and at ~0800 h, four basal blood samples were drawn for measurement of plasma glucose concentration and specific activity of insulin. Intravenous insulin infusion (Humulin; Lilly) diluted in 0.15 mol/liter saline containing 1% (w/v) human albumin was then begun at a rate of 80 milliunits · min−1 · kg−1. Blood samples were obtained every 5 min with a glucose analyzer (YSI 2700 analyzer; YSI, Yellow Springs, OH) for measurement of plasma glucose. A variable infusion of [3-3H]glucose-enriched 20% glucose was delivered to maintain a plasma glucose concentration of 5 mmol/liter. During the last 40 min of the insulin infusion, blood samples were obtained at 10-min intervals for determination of plasma glucose concentration and insulin. The study protocol was approved by the Human Subjects Committee of the University of California San Diego. This study was described in Ref. 10.

The cohort parameters of the individual studies are summarized in Table 1.
Whereas adiponectin levels are significantly reduced in states of decreased insulin sensitivity in humans under essentially all circumstances, insulin resistance in mice is often but not always associated with reduced adiponectin levels. This is particularly relevant for monogenic lesions such as the ones found in db/db and ob/ob mice. We have previously shown that db/db mice demonstrate comparable levels of circulating adiponectin as lean heterozygote littermates (9). To determine whether differences between these animals could partially be explained on the basis of differential distribution of adiponectin complexes in serum, we analyzed serum from male db/db and db/+ mice by velocity sedimentation followed by SDS-PAGE (Fig. 1A). Similar to our previous findings, lean and obese animals had comparable total levels of adiponectin circulating in serum (Fig. 1B). However, db/db mice demonstrate a significantly decreased percentage of adiponectin in the HMW form (Fig. 1C). Similar reductions in percentage of HMW adiponectin can be seen in a number of other diabetic mouse models, including the ob/ob mouse (not shown).

**Thiazolidinedione Treatment Affects Circulating HMW/LMW Adiponectin Complex Ratios in Mice and Humans**—TZD treatment leads to an induction of serum adiponectin and ameliorates the hyperglycemia, hypertriglyceridemia, and insulin resistance in the db/db mouse model within an 11-day course of treatment (9). To determine whether TZD treatment affects the relative circulating concentrations of adiponectin oligomers in serum, a cohort of male db/db mice was treated with rosiglitazone, and adiponectin complexes were analyzed by velocity sedimentation. Prior to treatment, adiponectin is predominantly found in the LMW (hexameric) form of adiponectin, consistent with values from wild type male mice. However, following 11 days of rosiglitazone treatment, the percentage of adiponectin found in the HMW form nearly doubled, to ~45% of total circulating adiponectin (Fig. 1D). Rosiglitazone treatment of ob/ob mice gave rise to similar induction in HMW adiponectin (not shown). Placebo treatment did not result in any significant change in adiponectin oligomeric distribution (not shown); nor did a 7-day treatment with PPARα agonist that was equally successful in reducing serum glucose, triglyceride, and insulin levels (by 45, 45, and 80%, respectively). This indicates that this shift in complex distribution can be attributed directly to TZD treatment and is not an indirect consequence of a systemic metabolic improvement (Fig. 1E).

In order to see if this relative improvement in HMW adiponectin can also be observed in human subjects treated with TZDs, we initially tested the effects in a cohort of nondiabetic males (Study A (9)). Since these were lean nondiabetic patients, no significant changes in fasting insulin or glucose were seen during the treatment period (2 weeks) with either rosiglitazone or placebo (not shown). Adiponectin complexes were analyzed in a double-blind fashion by velocity sedimentation before and after treatment (Fig. 2A). Only minor changes in total circulating adiponectin levels or in either adiponectin complex were seen in placebo-treated patients. Rosiglitazone-treated patients demonstrated significantly increased total adiponectin, primarily as a result of a dramatic increase in circulating HMW form (Fig. 2B). As a consequence, the HMW/total adiponectin ratio is significantly increased in rosiglitazone-treated individuals, with the post-treatment value reaching nearly 50% (Fig. 2C).

**Improvement in Insulin Sensitivity Post-TZD Treatment Correlates with Increased HMW/Total Ratio, Not with Total Adiponectin Levels**—To gain a better understanding of the functional relevance of the increased percentage of the HMW form in circulating adiponectin following thiazolidinedione treatment, we analyzed adiponectin complexes pre- and post-treatment in various cohorts of diabetic subjects. In one cohort, patients were treated with troglitazone for a period of 3 months (Study B (20)), and insulin sensitivity (SI) was determined before and after treatment. Insulin sensitivity of the group as a whole increased. Nearly all (12 of 13) subjects demonstrated an increase in circulating adiponectin levels, ranging from 20 to 150% above base-line levels. There was no significant correlation between the increase in total adiponectin levels and the improvement in insulin sensitivity (Fig. 3A), consistent with previous reports in the literature that suggest that although most patients demonstrate an increase in circulating adiponectin levels post-TZD treatment, these increased levels in individual patients do not correspond to the magnitude of increase in insulin sensitivity (10). In fact, in this particular cohort, approximately half of the patients demonstrated improvement in circulating levels of adiponectin by 25% or more without any significant change in insulin sensitivity. By contrast, there was good correlation between changes in insulin sensitivity and improvements in percentage of HMW/total adiponectin values (Fig. 3B). Similar to the trend observed in nondiabetic subjects, the absolute levels of HMW adiponectin increased in serum from most patients following thiazolidinedione treatment; however, there were type 2 diabetic patients who induced both total and HMW adiponectin levels to a similar degree and, thus, failed to improve their HMW/total ratio and demonstrated no improvement in insulin sensitivity. In order to not only account for absolute levels of HMW but to also take the total amount of adiponectin into consideration, we defined a new index ($S_A$) reflecting the relative contribution of the HMW complex to circulating adiponectin levels ($S_A = HMW/total$). $S_A$ rather than total adiponectin levels accounts for the patients in the cohort seen in Fig. 3A that do not demonstrate a quantifiable

### Table I

**Clinical characteristics of subjects examined in studies A–D**

| Study | Cohort examined | Average age (range) | Average BMI (range) | Fasting glucose ± mg/dl | Fasting insulin (units/liter) | Treatment |
|-------|-----------------|---------------------|---------------------|-------------------------|-------------------------------|-----------|
| A     | 20, nondiabetic males | 24 (18–42) | 24.4 ± 2.6 | 86 ± 7 | ND | Placebo |
| B     | 18, type 2 diabetic patients (14:4, male/female) | 66 (45–82) | 27 (20–38) | 180 ± 14 | 15.3 ± 2.8 | Rosiglitazone, 4 mg twice daily (2-week treatment) |
| C     | 20, female GDM patients, responders | 35 (24–48) | 28 (21–40) | 94 ± 8 | ND | Placebo |
| D     | 10, type 2 diabetic patients (9:1, male/female) | 51 ± 3 | 33.6 ± 2.0 | 178 ± 14 | 20.6 ± 4.6 | Rosiglitazone 400 mg/day (12-weeks treatment) |
|       | 9, obese nondiabetic patients (8:1, male/female) | 45 ± 2 | 34.8 ± 1.2 | 80 ± 2 | 16.0 ± 2.7 | Placebo |
|       | 8, lean (7:1, male/female) | 50 ± 2 | 24.3 ± 0.8 | 77 ± 2 | 4.3 ± 0.5 | Placebo |

* ND, not determined.

GDM, gestational diabetes mellitus.
improvement in insulin sensitivity, and the very tight correlation observed underlines the emerging finding that the proportion of adiponectin in the HMW form rather than absolute circulating levels may play an important role in the amelioration in insulin resistance following treatment with TZDs.

Increased Adiponectin Production Per Se Does Not Trigger an Increase in the Proportion of HMW/total adiponectin ratio post-TZD treatment. We observed better correlations with $S_A$ than with either induction of total or HMW adiponectin post-TZD treatment, leading to a model of competition between the HMW and LMW forms of adiponectin for activity, potentially through a mechanism related to receptor clustering. To confirm these initial results and to separate TZD responsiveness of adiponectin complexes from a potential "mass action" type response that would explain increased levels of HMW in serum simply on the basis of increased production or secretion of adiponectin at the level of the adipocyte, we analyzed an independent cohort of human subjects. Selection of participants from the Troglitazone in the Prevention of Diabetes (21) study (Study C) was based on either significant ($>50\%$) improvement in measures of insulin sensitivity following troglitazone treatment ($n=20$) or no significant ($<15\%$) change in $S_A$ post-treatment ($n=20$) in order to determine the extremes of clinical outcome post-TZD administration. High responders demonstrated, by definition, a large increase in insulin sensitivity compared with nonresponders, and as expected, there was a similar distinction between subgroups in $S_A$, a large increase in the responders and no significant change in nonresponders post-treatment (Fig. 4A). Total adiponectin levels increased in both subgroups, albeit to a lesser degree in nonresponders, suggesting dissociation between changes in total adiponectin and $S_A$, and in fact, changes in insulin sensitivity correlated only with improved $S_A$ and not change in total adiponectin levels (Fig. 4, B and C). Similar to previous studies, there were a number of instances of increased total adiponectin levels unaccompanied by improved insulin sensitivity. In this study, we also noted several patients who significantly improved insulin sensitivity without a concomitant increase in total adiponectin levels. By contrast, there are no patients who...
significantly change their SA without an improvement in insulin sensitivity, and vice versa. This study demonstrates that a shift in SA is possible without an increase in total adiponectin production levels, whereas an increase in total production levels does not necessarily result in an increased SA.

Increased SA Correlates with Improved Hepatic Insulin Sensitivity but Not with Improvement in Muscle Glucose Disposal Post-TZD Treatment—In each cohort examined, alterations in the HMW/total adiponectin ratio (SA) correlated with improvements in systemic insulin sensitivity in response to TZDs, as measured by changes in SI. However, it is not clear which tissues are primarily affected by the altered complex ratios. Previous studies have implicated both liver (decreased hepatic gluconeogenesis through inhibition of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase) (5) and muscle (increased fatty acid oxidation through inhibition of acetyl-CoA carboxylase) (22) as mediators of adiponectin activity. These same tissues have been implicated as targets of TZD action. We therefore correlated specific measures of hepatic insulin sensitivity, as determined under basal conditions, and insulin-stimulated glucose disposal in muscle upon hyperinsulinemic conditions with changes in SA before and after TZD treatment (Study D (10)). Patients with type 2 diabetes and age-matched nondiabetics were analyzed by hyperinsulinemic-euglycemic clamps before and after 3 months of troglitazone (600 mg/day) treatment. Induction of total adiponectin levels did not correlate with improved peripheral insulin sensitivity in diabetic patients as judged by improvement in hyperinsulinemia-induced glucose disposal by muscle (Rd values) (Fig. 5A); nor did total adiponectin values correlate with improvements in fast-
Increased fasting hepatic glucose output (FHGO) (Fig. 5B). Further, we failed to see a correlation between $S_A$ and $R_d$ (Fig. 5C). By contrast, there was a close correlation between changes in $S_A$ and decreased fasting hepatic glucose output (Fig. 5D). Importantly, in all three studies with detailed data on insulin sensitivity (studies B–D), focusing only on induction of total HMW complex (and not the ratio between HMW and total adiponectin) reveals significant correlations. However, the data summarized in Table II clearly demonstrates that $S_A$ is a considerably more powerful predictor of improvements in insulin sensitivity.

These human studies indicate that changes in $S_A$ quantitatively reflect improvements in insulin sensitivity in response to TZD treatment. Our initial observations in db/db mice suggest that in murine models, $S_A$ values at any given time may also be a better reflection of insulin sensitivity than total levels (Fig. 1) (7, 23). To test whether $S_A$ values in humans can be meaningful indicators of insulin sensitivity, we compared total adiponectin levels and $S_A$ values among three groups: (a) patients with type 2 diabetes, (b) BMI-matched obese patients with normal fasting glucose levels but with high fasting insulin levels comparable with the type 2 diabetic patients, and (c) lean individuals with normal glycemia and insulinemia. Total adiponectin levels were lowest in diabetics, intermediate in obese people with normal glucose tolerance, and highest in lean controls (Fig. 5E). $S_A$ values were similar in diabetic and obese individuals; although all three groups show increased $S_A$ with TZD treatment, insulin-resistant subjects demonstrated significantly lower $S_A$ values than lean controls (Fig. 5F). These findings are consistent with our observations in the murine models that hyperinsulinemia can trigger a specific disappearance of the HMW form and suggest that $S_A$ may be a more useful parameter than total adiponectin levels in the context of determining insulin sensitivity in humans as well.

**Intravenous Injection of HMW Adiponectin, but Not Hexameric Adiponectin, Leads to Decreased Serum Glucose**—Previous work in our laboratory has demonstrated that properly folded and assembled full-length adiponectin, when introduced into animals through either intraperitoneal or intravenous injection, leads to a significant decrease in serum glucose levels. To determine whether there is any evidence for differential biochemical activity of the HMW and LMW adiponectin complexes, we injected purified HMW (1 or 2 μg/g body weight) or LMW (2 μg/g body weight) into male animals. To avoid any confounding effects of various circulating endogenous complexes, these injections were performed in mice carrying a chromosomal deletion at the adiponectin locus that completely lack any endogenous circulating adiponectin.² HMW adiponectin dose-dependently reduces plasma glucose levels, whereas purified hexameric adiponectin lacks the ability to induce a decrease in plasma glucose levels compared with buffer injection (Fig. 6). Since male mice typically display about 80% of their adiponectin in the LMW form (corresponding to a 12–15-fold molar excess), solubility issues of purified complexes prevented us from injecting mixtures of the two complexes that would effectively mimic this extreme molar excess of LMW complexes.

**Adiponectin Complex Secretion Is Regulated at the Level of Adipose Tissue**—We have previously shown that iodinated adiponectin complexes are stable in serum and do not interconvert post-secretion. We have recently confirmed these observations in adiponectin knockout animals using nonderivatized, fully native adiponectin complexes (data not shown). Therefore, we hypothesized that the mechanism of increased HMW adiponectin post-TZD treatment was mediated by adipocytes, through differential secretion of the two oligomeric forms. We isolated various adipose tissues and serum from male and female mice, and analyzed by velocity sedimentation the complex distribu-

---

² M. Rajala, H. Chen, and P. E. Scherer, manuscript in preparation.
FIG. 5. Change in HMW/total adiponectin ratio is reflective of TZD-mediated improvements in hepatic insulin sensitivity and decreased basal $S_A$ is associated with the insulin-resistant state. Type 2 diabetic patients ($n = 10$) were analyzed by hyperinsulinemic-euglycemic clamps before and after 3 months of 600 mg/day troglitazone treatment. Basal (fasting) hepatic glucose output ($FHGO$) was measured by $[^3]H$glucose tracer studies, and muscle glucose disposal was assessed during infusion of 80 milliunits/m²/min insulin. Total adiponectin and complex levels were determined as before and correlated: percentage change of total adiponectin versus percentage increase of $R_d$ (A) (pre- and post-troglitazone: $r^2 = 0.153$) or percentage decrease of hepatic glucose output (B) ($r^2 = 0.012$) and percentage change of HMW/total adiponectin $\Delta S_A$ (C) versus percentage increase of $R_d$ ($r^2 = 0.397$, $p = 0.07$) or percentage decrease of hepatic glucose output (D) ($r^2 = 0.87$, $p = 0.003$). Total adiponectin levels pretreatment (E) and $S_A$ of diabetic patients (F) are compared with age- and BMI-matched hyperinsulinemic nondiabetic patients ($n = 9$) and age-matched lean patients ($n = 8$) and pre- (white bars) and post-treatment (black bars) values are graphed; the asterisks denote significant differences from the lean control group ($p < 0.05$).
tion from these animals. As previously reported, male and female mice display differential levels of adiponectin complexes in serum, with male animals displaying about 25% of their adiponectin in the HMW form, whereas female mice have slightly more than double that percentage (~50% HMW). Surprisingly, both males and females have similar proportions of HMW adiponectin within their adipose tissue; between 70 and 90% of adiponectin associated with adipose tissue is in the HMW form, in sharp contrast to the serum distribution within the same mice (Fig. 7A). The differences between tissue-associated and serum adiponectin ratios were quantitated and are associated and serum adiponectin ratios were quantitated and are higher in male animals than in female animals at all ages, and this ratio appears to be more pronounced in female animals than in male animals. As previously reported, male and female mice of both genders (Fig. 7B, C). A similar pattern was observed with human serum and adipose tissue (Fig. 7D). However, we did not see any significant changes in intracellular complex distribution in fat pads isolated from db/db animals either treated with TZD or treated with vehicle (data not shown). This suggests that the specific effects of TZDs on the HMW form could be the result of differential secretion of these complexes following TZD treatment or due to differential clearance of the complexes in TZD-treated animals. To determine whether TZD treatment could provoke differential release of adiponectin complexes in an isolated system, we treated 3T3-L1 adipocytes or H9c2-293T cells stably expressing murine adiponectin with rosiglitazone (or vehicle) for 24 h in serum-containing media and then allowed the cells to secrete into serum-free media for 16 h ± rosiglitazone. Both intracellular and secreted adiponectin shifted from a predominantly hexameric distribution to a more equal LMW/HMW ratio, suggesting a specific increase in assembly and secretion of the larger complexes (Fig. 7E and F). This increased HMW/total adiponectin ratio ($S_4$) could not be demonstrated in H9c2-293T cells, since both levels of total and HMW adiponectin were unchanged upon PPARγ agonist treatment. This demonstrates that the TZD effects on systemic adiponectin levels can be mimicked at the level of the adipocyte. Additional adipocyte-specific or -enriched PPARγ agonist-responsive factors are required for this process, since we cannot induce such a shift in HEK-293T cells.

**DISCUSSION**

We postulate a conceptually novel mechanism of regulation: alteration of oligomeric distribution of secreted adiponectin, leading to modulation of activity in target tissues. Our initial results suggested that db/db mice have a far lower percentage of circulating adiponectin in the HMW form despite similar total levels of the protein compared with phenotypically normal heterozygote and wild type littermates. This observation lends support to the hypothesis that levels of HMW adiponectin, not total circulating levels of the protein, may be a more relevant indicator of insulin sensitivity. We confirmed that diabetic patients have significantly decreased HMW/total adiponectin ratios compared with lean controls and defined a new index ($S_4$) that reflects this ratio.

The significance of decreased $S_4$ in mouse models of obesity and diabetes and in prediabetic and diabetic humans is underscored by the improvement of this ratio upon treatment with TZDs. TZDs appear to act by improving insulin sensitivity through binding and activation of PPARγ (24). Although there remains uncertainty about the exact site of action of TZDs, these drugs correct hyperglycemia and hyperinsulinemia in diabetic mouse models such as ob/ob and db/db mice or the Zucker fatty (fa/fa) rat, and they ameliorate insulin resistance in human type 2 diabetics. Many reports in the literature have documented an induction of serum adiponectin levels in mice and humans upon TZD treatment. With recent evidence sug-

**TABLE II**

| Study | Parameter | Correlation (HMW/total ratio) | Correlation (HMW only) |
|-------|-----------|------------------------------|-----------------------|
| B     | $S_1$     | Pearson = 0.91 ($p = 0.001$)  | Pearson = 0.76 ($p = 0.003$) |
| C     | $S_1$     | Spearman = 0.74 ($p = 0.004$) | Spearman = 0.38 ($p = 0.20$) |
| D     | Hepatic glucose output | Pearson = 0.85 ($p = 0.001$) | Pearson = 0.81 ($p = 0.001$) |

* Pearson coefficient, parametric (normality assumption) correlation.
* Spearman $p$-nonparametric, rank-based correlation estimate.

**FIG. 6.** Intravenous injection of HMW, but not hexameric adiponectin, leads to a dose-dependent decrease in serum glucose. Male adiponectin knockout animals of 10–12 weeks of age were injected via tail vein with 2 µg/g body weight HMW adiponectin, 2 µg/g LMW adiponectin, 1 µg/g HMW adiponectin, or buffer ($n = 6$ group), and serum glucose was assayed by glucometer at various times postinjection. Values that significantly differ from the buffer control are indicated by an asterisk ($p < 0.05$). Starting glucose levels, arbitrarily set to 100%, averaged 150 ± 5 mg/dl across all cohorts and spiked briefly at 30 min due to stress-induced by the intravenous injection procedure.
Fig. 7. HMW/total adiponectin ratios are significantly higher in adipose tissue compared with in serum. A, velocity sedimentation analysis followed by SDS-PAGE for four representative wild type C57/Bl6 female mice of serum and brown adipose tissue or gonadal adipose tissue. Six female (B) and six male (C) mice of between 8 and 10 weeks of age were subjected to the same analysis; despite a significantly lower percentage of serum HMW adiponectin in males, both male and female mice have similar HMW/total adiponectin (SA) ratios in adipose and significantly higher HMW/adiponectin ratios (SA) in adipose than in serum (BAT, brown adipose tissue; GAT, gonadal adipose tissue; IAT, inguinal adipose tissue). D, velocity sedimentation of human serum and subcutaneous abdominal adipose adiponectin from an obese, nondiabetic female patient. E, velocity sedimentation of secreted material from 3T3-L1 adipocytes or HEK 293T stably transfected with an expression plasmid encoding murine adiponectin with or without 1 μM rosiglitazone treatment. Each experiment was performed in triplicate, and a representative blot is shown. F, quantitation of the experiments shown in E. IC, intracellular; sec, secreted material.
sisting that some of the antidiabetic effects of TZDs require adipose tissue (14), much attention was focused on adiponectin and other adipokines as possible mediators of TZD effects, particularly with respect to TZD-mediated improvements on hepatic insulin sensitivity.

We find that changes in $S_A$ correlate only with TZD-mediated improvements in hepatic insulin sensitivity as assayed by basal hepatic glucose output and not with increases in glucose disposal by muscle under hyperinsulinemic conditions. This is consistent with previously demonstrated pharmacological effects of full-length adiponectin on liver (2, 5). Similar correlations with basal hepatic glucose output and increased $S_A$ are detectable in other type 2 diabetic populations in clamped settings, suggesting that basal hepatic glucose output under physiological insulinemia can be regulated by adiponectin. Furthermore, our work is in agreement with the phenotype of muscle-specific PPARγ knockout mice that demonstrate no TZD-mediated improvement in muscle insulin sensitivity but increased serum adiponectin as well as hepatic insulin sensitivity in response to TZD treatment (25). This suggests that adiponectin probably only mediates TZD antidiabetic effects in liver and may not be directly involved in the improvements seen in muscle. Thus, it may be surprising that we can also observe excellent correlations with improvements in $S_A$, an index that preferentially reflects contributions of muscle to a higher extent than contributions from the liver to overall insulin sensitivity in healthy patients (26). However, $S_A$ values in diabetic patients may reflect hepatic effects much more prominently due to the severe insulin resistance of muscle that does not significantly contribute toward glucose disposal under conventional clamp conditions (27).

How is this change in the ratio of circulating adiponectin complexes brought about? We observe a striking discrepancy between circulating and adipose tissue-associated adiponectin complexes, suggesting regulation at the level of secretion of the adiponectin complexes as a mechanism for regulation of adiponectin activity. Adipocytes, in response to paracrine or even autocrine signals, may release increased levels of HMW in response to TZD treatment or reduce secretion of the more active adiponectin complex in response to increased serum insulin concentration. The transcriptional profile of adipocytes stimulated by PPARγ agonists is more generally and dramatically altered, leading to increased production of factors, such as intracellular chaperones or additional required cofactors, that may be differentially involved in the maturation of adiponectin complexes in the secretory pathway. Current efforts in our laboratory focus on the identification of such effectors.

Additional important questions include whether increased levels of HMW adiponectin are a generally useful clinical parameter or only useful in the context of TZD-mediated effects. We demonstrate here that PPARγ agonist treatment, although resulting in similar metabolic improvement in db/db mice, does not lead to a significant change in the HMW/total adiponectin ratio, suggesting that $S_A$ induction is not simply reflective of improved systemic insulin sensitivity. Furthermore, the observation that $S_A$ is unaffected in this cohort supports a model that implicates a change in the HMW/total adiponectin ratio mechanistically in TZD-mediated improved insulin sensitivity. Preliminary results suggest that metformin-induced improvements in insulin sensitivity of diabetic patients are not accompanied by induction of $S_A$ (not shown). The scope of studies will have to be increased to determine whether improvement in insulin sensitivity achieved by other means, such as alternate pharmacological treatments or interventions such as diet/exercise-induced weight loss or gastric bypass surgery, also affect complex distribution.

We demonstrate in Table II that although significant correlations can be seen upon focusing on the induction of the HMW complex alone rather than the ratio of HMW/total adiponectin, correlations were consistently stronger with $S_A$ in each of the three independent cohorts of type 2 diabetic or prediabetic patients. Furthermore, the use of the ratio of HMW/Total could account for outliers seen when graphing either induction of total or HMW adiponectin levels. Based on the data analyzed thus far, $S_A$ offers a parameter superior to the measurements of total or HMW adiponectin alone and leads to the hypothesis that the HMW complex is the “active” adiponectin. Of interest in this context is the fact that the recombinant version of adiponectin that we have previously used in pharmacological experiments is primarily in the HMW form (not shown). The fact that the TZD-mediated increase of the HMW complex correlates with improved insulin sensitivity in liver rather than muscle is therefore fully consistent and may present a mechanistic rationale for the previously reported effects. Although speculative, the combination of correlational and pharmacological data suggests that a model of competitive antagonism between adiponectin oligomeric complexes is conceptually plausible, but further experiments will be required to directly evaluate this hypothesis.

Our previous data have indicated that recombinant adiponectin with a mutated cysteine 39, the residue critically involved in disulfide bonding and higher order complex formation, is proteolytically cleaved and more bioactive in vivo than the wild type counterpart (17). This ligand is monovalent yet represses hepatic glucose genesis at concentrations lower than wild type protein. This suggests an alternative model in which the clustering effect is not required to induce signaling directly but rather to initiate the induction of a reductase activity that breaks the HMW form into basic trimers that may then be substrates for a proteolytic processing step, leading to the generation of the active ligand. Indeed, we have previously provided evidence for a membrane-associated protease on the surface of hepatocytes, HEK 293, and other cell types (17). Reduction of serum factors coupled with proteolysis as a mechanistic route to ligand activation is emerging as a common theme in a number of secreted proteins, such as the processing of thrombospondin-1 and plasmin conversion to angiostatin (reviewed in Ref. 28). During the preparation of this manuscript, Waki et al. (29) published evidence demonstrating that serum from human subjects heterozygous for a rare G90R mutation in adiponectin was deficient in HMW adiponectin. The mutation of this key glycine residue in the collagenous domain of adiponectin led to diminished HMW secretion in vitro and in vivo. Interestingly, these mutations are associated with increased risk for type 2 diabetes in patients, lending further support for our hypothesis that the HMW complex is the bioactive adiponectin responsible for increased insulin sensitivity.

In the recent past, numerous studies have been published that measure total serum adiponectin and correlate total circulating levels of the protein with various clinical parameters, such as markers of inflammation, obesity, diabetes, and atherosclerosis. We suggest that the measurement of total serum adiponectin levels should continue to be used as a comparative assessment of insulin sensitivity. However, $S_A$ measurements are useful in cases where differences in insulin sensitivity cannot be fully explained on the basis of differences of absolute serum adiponectin levels alone.

Acknowledgments—We thank members of the Scherer laboratory for valuable comments, Ja-Young Kim for secretion experiments, Melissa Fazzari for statistical analysis, Alan Adams for the synthesis of the

---

3 M. Hawkins, unpublished observations.
Complex Formation and Bioactivity of Adiponectin

PPAR agonist (compound 10), and Surahb Patel for assistance in gel filtration chromatography. In addition, we acknowledge the helpful comments from Drs. David Moller, Luciano Rossetti, Ulrich Schubart, Elliot Goodman, and Michael Brownlee during the various stages of this project.

REFERENCES
1. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) Nature 372, 425–432
2. Berg, A. H., Combs, T., Du, X., Brownlee, M., and Scherer, P. E. (2001) Nat. Med. 7, 947–953
3. 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
4. Rajala, M. W., Obici, S., Scherer, P. E., and Rossetti, L. (2003) J. Clin. Invest. 111, 225–230
5. Combs, T. P., Berg, A. H., Obici, S., Scherer, P. E., and Rossetti, L. (2001) J. Clin. Invest. 108, 1875–1881
6. Vasseur, F., Lepretre, F., Lacquemant, C., and Froguel, P. (2003) Curr. Diab. Rep. 3, 151–158
7. Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuozawa, Y., Pratley, R. E., and Tataranni, P. A. (2001) J. Clin. Endocrinol. Metab. 86, 1930–1935
8. Coop, M., Havel, P. J., Uttschneider, K. M., Carr, D. B., Sinha, M. K., Boyko, E. J., Retzlaff, B. M., Knopp, R. H., Brunzell, J. D., and Kahn, S. E. (2003) Diabetologia 46, 459–469
9. 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. S., Chatterjee, K., Weiss, S., Larson, P. J., Gottesdiener, K. M., Gertz, B. G., Charron, M. J., Scherer, P. E., and Moller, D. E., and Jones, A. (2003) Bioorg. Med. Chem. Lett. 13, 3185–3190
10. Hevener, A. L., Engle, J., Brownlee, M., and Scherer, P. E. (2003) J. Biol. Chem. 278, 9073–9085
11. Scherer, P. E., Lisanti, M. P., Baldini, G., Sargiacomo, M., Corley-Mastick, C., and Lodish, H. F. (1994) J. Cell Biol. 127, 1233–1243
12. Day, C. (1999) Diabet. Med. 16, 179–192
13. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Khewer, S. A. (1995) J. Biol. Chem. 270, 12953–12956
14. Chao, L., Marcus-Samuels, B., Mason, M. M., Moitra, J., Vinson, C., Arioglu, E., Gavrilova, O., and Reitman, M. L. (2000) J. Clin. Invest. 106, 1221–1228
15. Kim, J. K., Fillmore, J. J., Gavrilova, O., Chao, L., Higashimori, T., Choi, H., Kim, H. J., Yu, C., Chen, Y., Qu, X., Haluzik, M., Reitman, M. L., and Shulman, G. I. (2003) Diabetes 52, 1311–1318
16. Olefsky, J. M. (2000) J. Clin. Invest. 106, 467–472
17. Pajvani, U. B., Du, X., Combs, T. P., Berg, A. H., Rajala, M. W., Schulthess, T., Engel, J., Brownlee, M., and Scherer, P. E. (2003) J. Biol. Chem. 278, 9073–9085
18. Scherer, P. E., Lisanti, M. P., Baldini, G., Sargiacomo, M., Corley-Mastick, C., and Lodish, H. F. (1994) J. Cell Biol. 127, 1233–1243
19. Adams, A. D., Hu, Z., von Langen, D., Dadiz, A., Elbrecht, A., MacNaul, K. L., Berger, J. P., Zhou, G., Doebber, T. W., Meurer, B., Forrest, M. J., Moller, D. E., and Jones, A. B. (2003) Bioorg. Med. Chem. Lett. 13, 3185–3190
20. Prigeon, R. L., Kahn, S. E., and Porte, Jr., J. (1996) J. Clin. Endocrinol. Metab. 83, 819–823
21. Azé, S. P., Peters, R. K., Berkowitz, K., Kjems, S., Xiang, A., and Buchanan, T. A. (1998) Control Clin. Trials 19, 217–231
22. 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, R., Horie, M., Takeda, S., Aoyama, T., Funahashi, T., and Matsuzawa, Y. (2002) Nat. Med. 8, 731–737
23. Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishiida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T., and Matsuzawa, Y. (1999) Biochem. Biophys. Res. Commun. 257, 79–83
24. Forman, B. M., Tontoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) Cell 82, 803–812
25. Hevener, A. L., Hu, Z., Barak, Y., Le, J., Bandyopadhyay, G., Olson, P., Wilkes, J., Evans, R. M., and Olefsky, J. (2003) Nat. Med. 9, 1491–1497
26. Bergman, R. N., Prager, R., Volund, A., and Olefsky, J. M. (1987) J. Clin. Invest. 79, 780–800
27. Vaag, A., Alford, F., Henrikson, F. L., Christopher, M., and Beck-Nielsen, H. (1995) Diabetologia 38, 326–336
28. Hogg, P. J. (2003) Trends Biochem. Sci. 28, 210–214
29. Prigeon, R. L., Kahn, S. E., and Porte, Jr., J. (1996) J. Clin. Endocrinol. Metab. 83, 819–823