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High-resolution identification of human adiponectin oligomers and regulation by pioglitazone in type 2 diabetic patients

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ABSTRACT

Adiponectin is an adipokine with insulin-sensitizing, anti-inflammatory, and cardiac protective actions. It homo-oligomerizes into trimers, hexamers, and higher molecular weight (HMW) species, which are not fully characterized. We describe high-resolution separation of adiponectin oligomers under native conditions in polyacrylamide gel coupled with methods for producing standards to provide facile and accurate identification of the oligomers. Using these procedures, adiponectin trimers in human and rodent plasma were found to migrate as two distinct populations. Distributions of these two populations are linearly proportional in plasma from type 2 diabetic patients before ($R^2 = 0.903$, $P < 0.001$) and after ($R^2 = 0.960$, $P < 0.0001$) 12 weeks of treatment with pioglitazone as well as from control subjects ($R^2 = 0.891$, $P < 0.0001$). In addition, HMW adiponectin could be separated into three distinct oligomers: nonamer (9mer), dodecamer (12mer), and the previously characterized octadecamer (18mer). Plasma concentrations of all oligomers increased on pioglitazone treatment, with the largest fold increase being observed in 9mers and 12mers compared with baseline. Increasing concentrations of adiponectin during oligomerization in vitro led to a disproportionate increase in 18mers. The difference between in vivo and in vitro observations suggests that higher total adiponectin protein concentration contributes to pioglitazone's ability to enhance HMW adiponectin levels, but additional factors likely affect oligomer assembly or turnover independently.

Adiponectin is a hormone produced mainly by adipocytes with positive or protective effects on insulin action, cardiac functions, and vasculature [1–5]. High levels of adiponectin are consistently associated with decreased risk for type 2 diabetes across many studies [6], whereas low levels of circulating adiponectin are associated with insulin resistance, coronary artery disease, and obesity [7–9]. Treatment with the thiazolidinedione (TZD)[2] class of insulin-sensitizing compounds results in elevated blood adiponectin [10]. Circulating adiponectin concentration has been proposed as a biomarker for metabolic disorders and glycemic control improvement [11,12]. As a result, it is important to understand the mechanisms that govern the concentration of adiponectin in circulation.

In rodents and humans, adiponectin circulates as several homo-oligomeric species. The smallest mature adiponectin oligomer is a homo-trimer that consists of a glycosylated collagen-like triple helical domain [7,13,14] and a globular head domain with a highly hydrophobic inner core formed by the interface of all three monomers [15]. The hexamer (6mer) is composed of two trimers arranged in a head-to-head, tail-to-tail orientation [16]. Larger adiponectin oligomeric species are readily observed in serum [13,17–19]. These species are collectively and commonly referred to as higher molecular weight (HMW) adiponectin, a term that reflects the paucity of information on their biochemical composition. The predominant HMW species has been determined to consist of 18mers [20,21].

Although the level of circulating adiponectin is closely associated with insulin sensitivity, the degree of association appears to be stronger with HMW adiponectin than with total adiponectin [22–29]. In particular, the S_a index, defined as the ratio of HMW to the sum of HMW and lower molecular weight (LMW) abundance in sucrose velocity gradient [22], has been shown to

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[2] *Abbreviations used:* TZD, thiazolidinedione; HMW, higher molecular weight; LMW, lower molecular weight; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; SEC, size exclusion chromatography; PBS, phosphate-buffered saline; FPG, fasting plasma glucose; Fx, fraction; ANOVA, analysis of variance; HSD, honestly significant difference.

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be useful in understanding the relationship between changes in HMW adiponectin and improvement in insulin sensitivity following TZD [22,28] or exercise training intervention [30] and overall risk for type 2 diabetes [31]. In principle, HMW adiponectin may be a better biomarker than total adiponectin. However, a major difficulty in using HMW adiponectin as a biomarker is that its biochemical composition remains poorly defined. HMW adiponectin appears to consist of several distinct species [17–19,32]. In fact, the ambiguity of what constitutes HMW adiponectin may underlie the controversy regarding whether HMW adiponectin or the S_a index is superior to total adiponectin as a biomarker of insulin action [33,34]. Many techniques have been used to distinguish different oligomeric species of adiponectin, including gel filtration chromatography [18,35], velocity sedimentation [22,36], gel electrophoresis [9,18,19,37–44], and isoform-specific enzyme-linked immunosorbent assay (ELISA) [45–47]. Although none of these is able to resolve different HMW isoforms with sufficient resolution, native gel electrophoresis appears to be most promising.

We have employed an improved native gel electrophoresis procedure based on a Tris–acetate buffer system to separate purified bovine adiponectin oligomers. Using this technique, we previously observed clearly defined intermediate oligomers between 6mers and 18mers [21,48,49]. Using 18mers and 6mers whose oligomerization states were independently determined by equilibrium sedimentation or mass spectrometry as standards, 9mers and 12mers were identified by plotting gel migration distance against natural logarithm of molecular weight. This procedure was also employed here to examine oligomerization states of adiponectin in plasma from normal individuals and diabetic patients before and after pioglitazone treatment.

Materials and methods

Native and denaturing electrophoresis of adiponectin

Samples for native polyacrylamide gel electrophoresis (PAGE) and nonreducing sodium dodecyl sulfate (SDS)–PAGE were prepared and separated in 7% Tris–acetate and 11% Tris–glycine–SDS gels, respectively, as documented previously [21]. For reducing SDS–PAGE, the samples were treated as for nonreducing denaturing electrophoresis except that 50 mM dithiothreitol (DTT) was included. Native and denaturing gels were stained by Coomassie Brilliant Blue G-250 and scanned in a LI-COR Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA) to quantify band intensity, as reported previously [50]. Ferritin, thyroglobulin, and catalase purchased from Sigma (St. Louis, MO, USA) or Amersham/GE Healthcare Life Sciences (Piscataway, NJ, USA) were run alongside purified adiponectin oligomers as molecular weight markers.

Immunoblot analysis of adiponectin oligomers

To assess adiponectin oligomerization states under native conditions, samples for analysis were diluted with concentrated native loading buffer to a final composition of 31 mM Tris (pH 6.8), 12% glycerol, and 0.05% Orange G prior to gel loading. The volumes of various types of samples loaded per lane are as follows: human plasma, mouse plasma and serum, fetal calf serum, and calf serum, 1 μl; size exclusion chromatography (SEC) fractions or conditioned media from 3T3-L1 adipocytes, 10 μl; calf serum clarified using protein G and Cibacron blue 3GA agarose beads with or without low pH treatment, approximately 20 μl. Details regarding human subjects from which plasma were obtained are described below in the "Subjects" section. Mouse plasma and serum were collected through tail veins from 3-month-old male C57Bl6/J mice with clotting allowed to take place at room temperature for 30 min to obtain serum. Calf serum and fetal calf serum were purchased from Invitrogen (Carlsbad, CA, USA) and Atlanta Biologicals (Lawrenceville, GA, USA), respectively. 3T3-L1 adipocytes were differentiated as described previously [18], and 7-day differentiated adipocytes were washed and incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) with 0.1% bovine serum albumin for 16 h prior to collection of conditioned media. Native immunoblot analysis of adiponectin was performed by transferring fractionated protein under native conditions in 7% Tris–acetate gels to nitrocellulose membranes at either 25 V overnight in a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA, USA) or 1 mAmp per cm^2 gel area for 90 min in a semi-dry transfer apparatus (Hoefer, Holliston, MA, USA) in 25 mM Tris (pH 8.3), 192 mM glycine, and 15% methanol. Membranes were blocked in Tris buffer saline supplemented with 5% nonfat dried milk (pH 7.5) and probed overnight with a rabbit antiserum raised against recombinant globular adiponectin (expressed and purified as described previously [51]) diluted 1:4000 in the same blocking buffer. Membranes were then washed and incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a final concentration of 1:5000. After extensive washing, the membranes were treated with chemiluminescent substrate (Pierce/Thermo Fisher, Rockford, IL, USA) and exposed to X-ray films. After the films are digitally scanned, band intensities were determined using the gel analysis module of ImageJ software to generate profiles of individual lanes and to measure areas under the peaks. Migration distances of individual bands from wells were measured with a ruler except when migration distances of diffuse trimers needed to be determined. In those cases, the distances of all oligomers were measured as numbers of pixels between wells and the peak positions of each band on lane profiles were generated by ImageJ software. Ferritin (Sigma) was used as molecular weight in native gels due to its orange color under native conditions. On transfer to nitrocellulose filter, the position of ferritin was marked with a pencil because its color fades during blocking and washing. The institutional animal care and use committee of University of Arizona, in accordance with Public Health Service animal welfare policy, approved all animal experiment protocols.

Purification of bovine adiponectin oligomers

Adiponectin was purified from calf serum (Invitrogen) or fetal calf serum (Atlanta Biologicals) as described previously [21]. Purified bovine adiponectin consists primarily of 18mers [20,21]. Minor amounts of 6mers that coeluted with 18mers in anion exchange chromatography [20,21] were separated from 18mers using a 16/60 Superdex 200 gel filtration column (GE Healthcare Life Sciences).

Size exclusion chromatography

Human plasma (1.3 ml) or pooled male Sprague–Dawley rat plasma (1 ml) was passed through a 0.45-μm syringe filter and loaded onto a Superdex 200 16/60 Hi-Load column (GE Healthcare) preequilibrated with phosphate-buffered saline (PBS). Fractions were collected in 0.6- or 0.5-ml volumes.

Mass spectrometry of adiponectin

Purified HMW adiponectin 18mers and glycine-treated adiponectin (both 1.5 mg/ml) were exchanged into 200 mM ammonium acetate buffer (pH 7.0) using centrifugal size exclusion columns (Bio-Rad). Samples were ionized via the nano-electrospray method and analyzed in a Synapt G2 quadrupole ion mobility time-of-flight mass spectrometer (Waters, Manchester, UK). Ion mobility mode

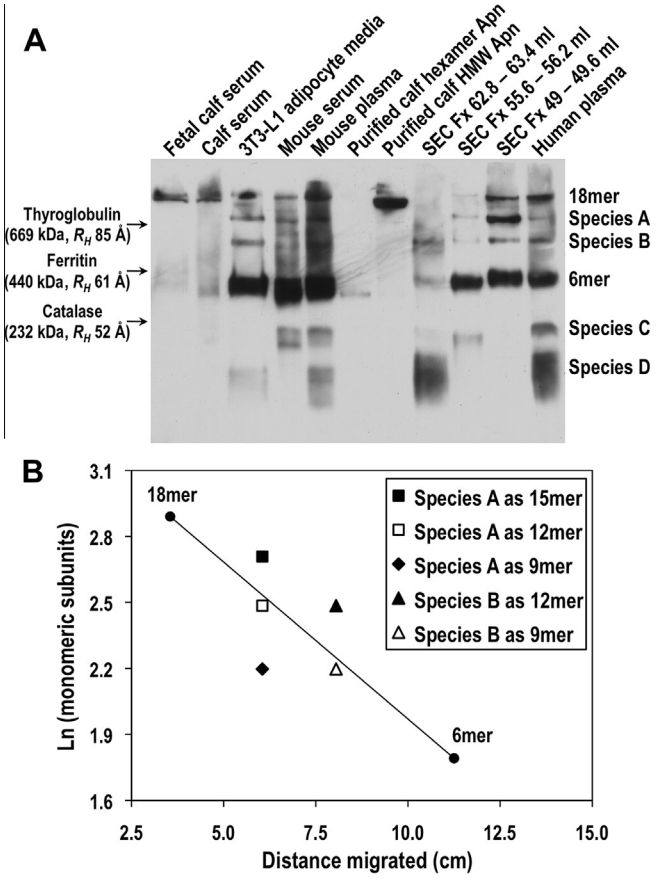

Fig.1. Analysis of adiponectin oligomers in complex biological mixtures using Western blot analysis following fractionation in 7% Tris–acetate native gels by electrophoresis. (A) Western blot analysis of adiponectin from various sources (right to left): human plasma, human plasma fractionated using SEC, pure adiponectin 18mers isolated from calf serum, pure 6mer adiponectin isolated from calf serum, mouse plasma, mouse serum, conditioned media from 3T3-L1 adipocytes, calf serum, and fetal calf serum. Nitrocellulose membranes were probed overnight with rabbit antiserum raised against recombinant globular adiponectin. Positions of the three native molecular weight markers—thyroglobulin, ferritin, and catalase—were established by running alongside purified adiponectin oligomers followed by staining in Coomassie Brilliant Blue G-250. For each marker, both the molecular weight and the Stokes radius (R_H in angstrom, Å) are shown to underscore the mismatch between adiponectin oligomers' actual molecular weight determined by equilibrium sedimentation or mass spectrometry and Stokes radii (~480 kDa and 90 Å, respectively, for 18mers) [20,21]. Apn, adiponectin. (B) Identification of oligomerization states of human adiponectin oligomers labeled species A and B. Migration distances of 18mers and 6mers in human plasma were plotted against natural logarithm of their subunit numbers with a straight line drawn through those two points. Migration distances of species A and B were then plotted against the natural logarithm of the five possible combinations of their subunit numbers: 15, 12, or 9 for species A and 12 or 9 for species B.

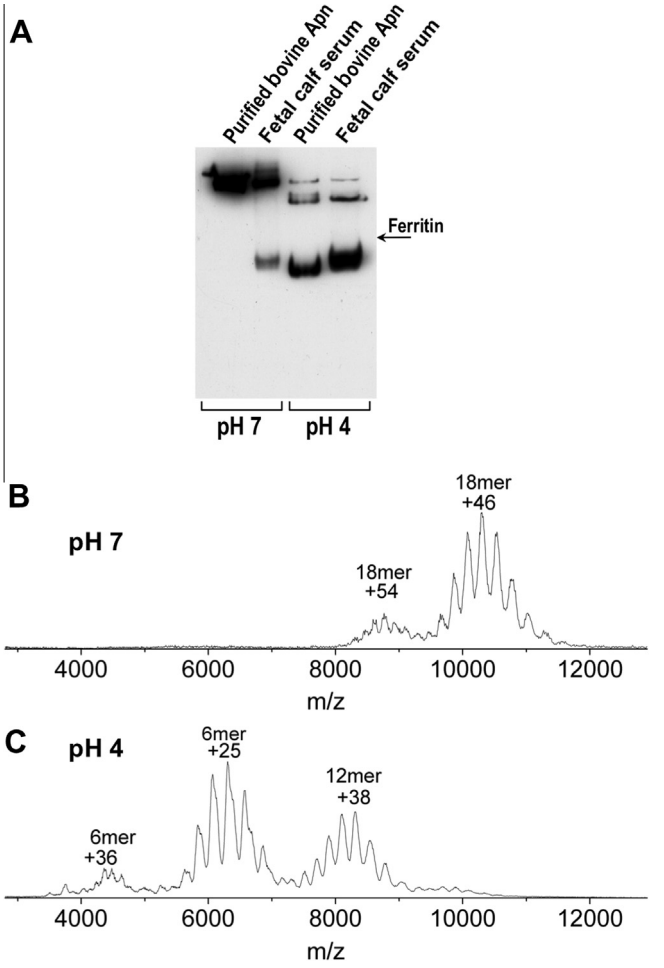

Fig.2. Fetal bovine serum at pHs 7.0 and 4.0 as standards for adiponectin oligomers in Western blot analysis. (A) The 7% Tris–acetate native gel electrophoresis of purified bovine adiponectin and fetal bovine serum before and after treatment in glycine buffer at pH 4.0. Fetal bovine serum was first clarified of excess immunoglobulins and albumin as described in Materials and Methods. Glycine (1 M) was added to clarified fetal bovine serum and pure adiponectin from calf serum to lower pH to 4.0 as described in Materials and Methods. Apn, adiponectin. (B and C) To confirm the oligomerization states of purified bovine adiponectin, mass spectra of adiponectin on nano-electrospray ionization were obtained before (B) and after (C) treatment in glycine buffer at pH 4.0. The predominant species of the sample at pH 7.0 was the 18mer, which has a major charge state distribution centered around +46 and a minor charge state distribution centered around +54. Both series correspond to a mass of 473.5 kDa (26162 ± 264 Da per protomer, as determined by matrix-assisted laser desorption/ionization [MALDI]; data not shown). Following pH 4.0 treatment, the major oligomer was the 6mer (157.6 kDa), which also has major and minor charge state distributions. Another oligomeric species, the 12mer (315.8 kDa) with a single charge state distribution centered around +38, was also present at lower abundance.

was not used for the data shown here. The spraying voltage in the capillary was 1.5 kV. The cone voltage used was 100 V. The backing pressure in the source was 5.0 mbar, and the pressure in the time-of-flight analyzer was 7.8×10^{-7} mbar. Spectra were visualized using Waters MassLynx version 4.1 software. Charge states of the peaks were assigned manually.

Preparation of octadecameric and hexameric adiponectin standards and conversion of purified adiponectin 18mers to 6mers and trimers

Fetal calf serum (Invitrogen) was added to protein G or protein A/G agarose (Pierce/Thermo Fisher) resin to 2.5:1 (v/v) and incubated at 4 °C overnight in a rotator to decrease the immunoglobulin concentration. The resin was preequilibrated by washing and resuspending 1:1 (v/v) in 25 mM Tris (pH 7.2) and 150 mM NaCl. The protein G resin was pelleted by centrifugation, and the supernatant was then added to Cibacron Blue 3GA agarose (Sigma–Aldrich, St. Louis, MO, USA) resin (washed and suspended at a 1:1 ratio in PBS) at 1.9:1 (v/v) and incubated for 30 min at room temperature in a rotator to remove excess albumin. The resins were regenerated according to manufacturer's instructions prior to reuse. The predominant adiponectin isoform in calf serum is 18mers and was used without further treatment as the 18mer standard. To convert 18mers in resin-treated calf serum to 6mers, it was first diluted 1:1 (v/v) with PBS, and 1 M glycine (pH 3.0 or 2.5) was subsequently added to adjust final pH to 4.0 as judged by pH color indicator strips. The reactions were incubated at room temperature for 1 h, followed by the addition of 1 M glycine at pH 10.0 to return the reactions' pH to 7.0. Native sample loading buffer was then added, and gel loading was performed as described above. Purified adiponectin 18mers

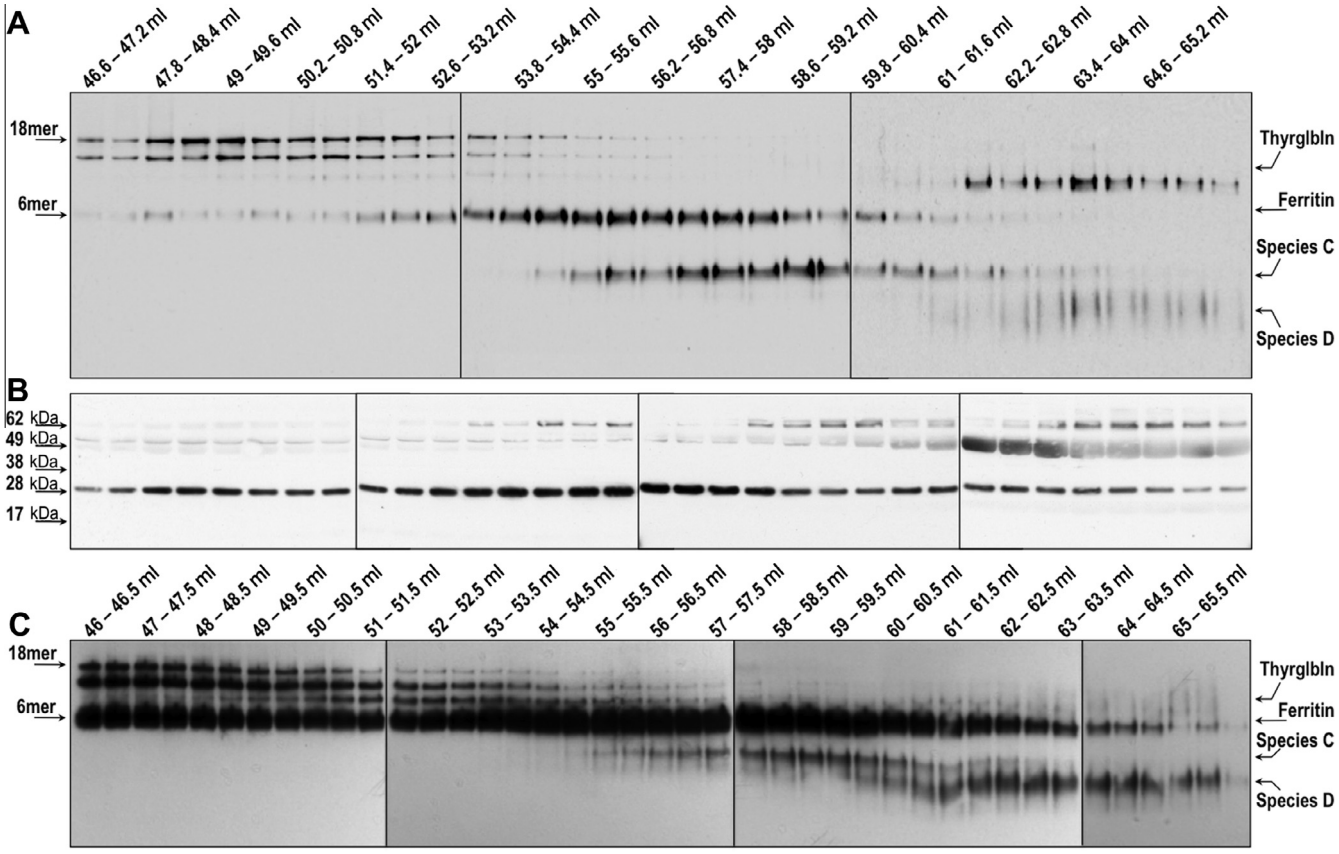

Fig.3. SEC elution profile of adiponectin oligomers in human plasma (A and B) and rat plasma (C) as determined by native (A and C) and denaturing and reducing (B) immunoblot analysis. Approximately 1.3 ml of human plasma and 1 ml of male Sprague–Dawley rat plasma were injected into a PBS-equilibrated 16/60 Superdex 200 column with a void volume of approximately 45 ml. Fractions were collected in 0.6- and 0.5-ml volumes for human and rat plasma, respectively. Each lane of the gel was loaded with 15 μl of eluted fraction. Every other lane is labeled with the elution volume of the fraction. Bands corresponding to trimer exhibit differential migration profiles, as seen in fractions 55–61 ml (discrete trimer, species C) and fractions 61–65 ml (diffuse trimer, species D). Nitrocellulose membranes were probed overnight, with rabbit antiserum was raised against recombinant globular adiponectin. Thyrglbln, thyroglobulin.

were treated identically as calf serum with reduced immunoglobulin and albumin content for conversion to 6mers. To convert purified 18mers to trimers, samples were first treated with 50 mM DTT for 30 min at room temperature prior to the glycine treatment.

Sialidase treatment

Human plasma and SEC fractions of human plasma were treated with Glyko Sialidase A (GK80040, Prozyme, Hayward, CA, USA) at 37 °C for 1 h according to the manufacturer's protocol.

Subjects

Men and women (ages 35–75 years) with type 2 diabetes as defined by either fasting plasma glucose (FPG) > 126 mg/dl at entry or FPG > 115 mg/dl and a 2-h oral glucose tolerance test (O-GTT) glucose > 200 mg/dl were eligible. FPG at entry needed to be <200 mg/dl. For women, use of adequate contraceptive control was required. This could include oral contraceptives, hysterectomy, tubal ligation, or postmenopausal status, as defined by >6 months without a menstrual cycle and follicle-stimulating hormone (FSH) > 40 mIU/ml. Patients were excluded if they had significant renal, cardiac, liver, lung, or neurological disease, although controlled hypertension was acceptable if baseline blood pressure was <140/90 on medications. Patients with prior use of TZDs (troglitazone, rosiglitazone, or pioglitazone) or beta blockers, patients currently pregnant, smokers, and patients with alcohol or other drug abuse or who were unwilling to abstain from caffeine for 48 h and alcohol for 24 h prior to metabolic rate measurements were also excluded. Subjects with liver function tests at baseline (AST/ALT/GGT or alkaline phosphatase) >2.5 times the upper limit of normal were not included. Patients taking drugs known to affect lipid metabolism, energy metabolism, or body weight, such as orlistat, sibutramine, ephedrine, phenylpropanolamine (Dexatrim), and corticosteroids, were excluded from the study. The plasma samples analyzed in this study were randomly selected from participants in a larger trial [52].

Pioglitazone study protocol

Patients received the usual care for their diabetes with instructions on a healthy diabetic diet by a dietician. Blood glucose monitoring was encouraged. The hemoglobin A1C target was <7.0. Pioglitazone and matching placebos were prepared by Takeda Pharmaceuticals and were given as a single daily dose of 30 mg/day or placebo each morning. If after 8 weeks the hemoglobin A1C was >7.0 or FPG > 100 mg/dl, the dose of pioglitazone was increased to 45 mg/day, which occurred in all but 1 participant. If individuals had an increase in the hemoglobin A1C > 12% or an increase in FPG > 240 mg/dl, they were to be treated with sulfonylureas or insulin, but this was not necessary in the current study. Subjects on sulfonylureas or metformin who experienced hypoglycemia had the dose of these medications reduced or the medication discontinued. Patients visited the clinic weekly for 4 weeks and each month thereafter. During these visits, blood pressure was measured and a blood sample was collected for determination of hemoglobin A1C, fasting glucose (week 8 and end of study), serum lipids, liver function tests, and adiponectin. Plasma was collected from a reference population

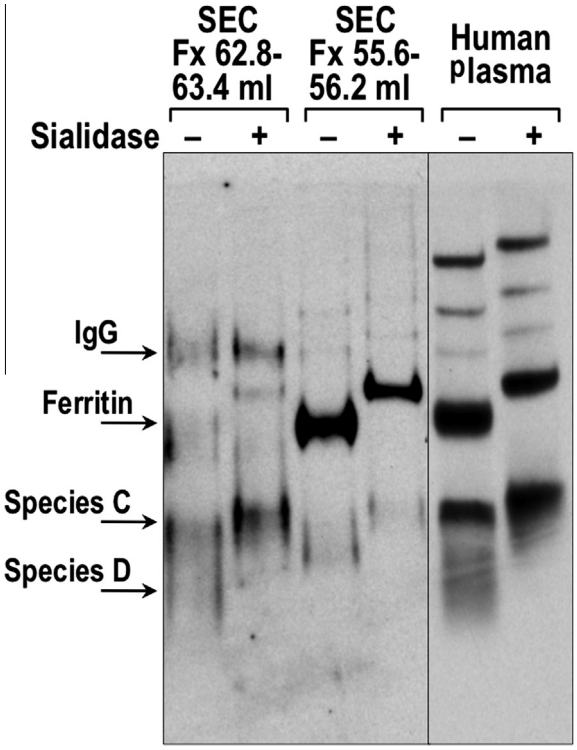

Fig.4. Migration of adiponectin oligomers in human plasma following the removal of posttranslationally attached sialic acid residues. Human plasma and SEC fractions of human plasma were treated with or without sialidase at 37 °C for 1 h prior to fractionation in 7% Tris–acetate native gels. Western blotting was performed using rabbit antiserum raised against recombinant globular adiponectin. IgG, immunoglobulin G.

consisting of healthy nondiabetic female and male subjects. Participants gave written informed consent, and the study was approved by the institutional review board of the Pennington Biomedical Research Center.

Reassembly of adiponectin oligomers in vitro

Purified HMW adiponectin (10 μM monomer) was treated with 10 mM DTT at 37 °C for 1 h, followed by adjustment of pH to 4.0 in 50 mM glycine to generate trimers. The reactions were then dialyzed against PBS in an anaerobic chamber for 3 to 4 h to lower DTT and glycine concentrations and to return to neutral pH. After dialysis, the adiponectin samples were serially diluted to three different trimer concentrations—2.67, 1.33, and 0.67 μM—and allowed to reoligomerize in the presence of 3 mM H_2O_2 for 45 min at 25 °C. Hydrogen peroxide was directly added to the samples rather than introduced slowly through dialysis to facilitate rapid reoligomerization. Reactions were quenched in 3 mM *N*-ethylmaleimide and frozen until gel electrophoresis. Equal amounts of adiponectin were loaded onto gels to ease comparison among samples with different protein concentrations.

Statistical analysis

Data are presented as means ± standard errors unless otherwise noted. Statistical comparisons between one treatment group and the corresponding control group were performed using Student's paired *t* test (two-tailed). In experiments with more than one treatment group, the differences between different groups were assessed using one-way analysis of variance (ANOVA) with Tukey's HSD (honestly significant difference) post hoc test. The particular statistical analyses that applied to the results in specific experiments are described in figure legends. For all experiments, representative gels are shown and were repeated at least three times unless noted otherwise.

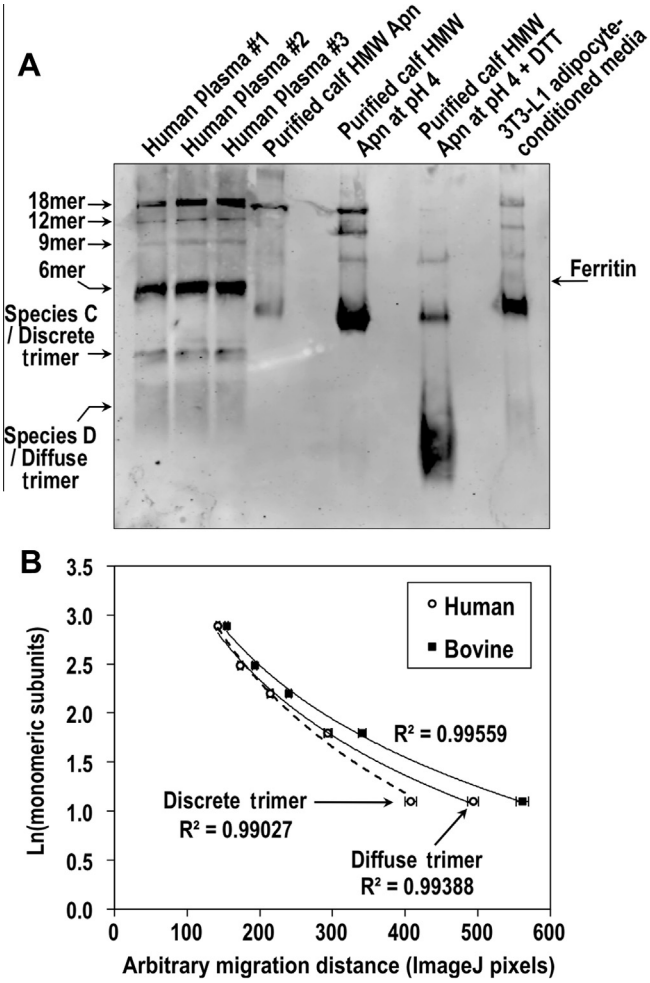

Fig.5. Direct comparison of human plasma adiponectin oligomers with those secreted from 3T3-L1 adipocytes and 6mers and trimers prepared from collapse of purified calf 18mers using native immunoblotting. Bovine 6mers and trimers were obtained by lowering the pH of purified 18mers to 4.0 in the absence and presence, respectively, of 50 mM DTT as described in Materials and Methods. Human plasma, 3T3-L1 adipocyte-conditioned media, purified calf 18mers, and 18mers collapsed to 6mers and trimers were loaded onto 7% Tris–acetate gels. Following transfer to nitrocellulose filters, Western blotting was performed using rabbit antiserum raised against recombinant globular adiponectin, and results are shown in panel A. Apn, adiponectin. Profiles of each lane are plotted using the gel analysis module of ImageJ, and distances between the peaks of each band and the wells are measured as pixels on the digitized images. The migration distance of each band (mean ± standard deviation from two independent experiments) in lanes with human plasma or untreated/treated purified calf HMW adiponectin is plotted in panel B against the natural logarithm of the subunit number of the corresponding oligomer. Logarithmic regression lines are plotted through bovine (solid squares) and human (open circles) 18mers, 12mers, 9mers, 6mers, and trimers. Separate logarithmic regression lines are drawn for diffuse trimers (solid line) and discrete trimers (dashed line), and the transformed regression model R^2 values for each curve are shown.

Results

Migration profiles of adiponectin oligomers from plasma or serum in native gel

To understand the role of adiponectin in physiological processes, it is necessary to assess oligomerization states of adiponectin with a high degree of resolution in large numbers of biological

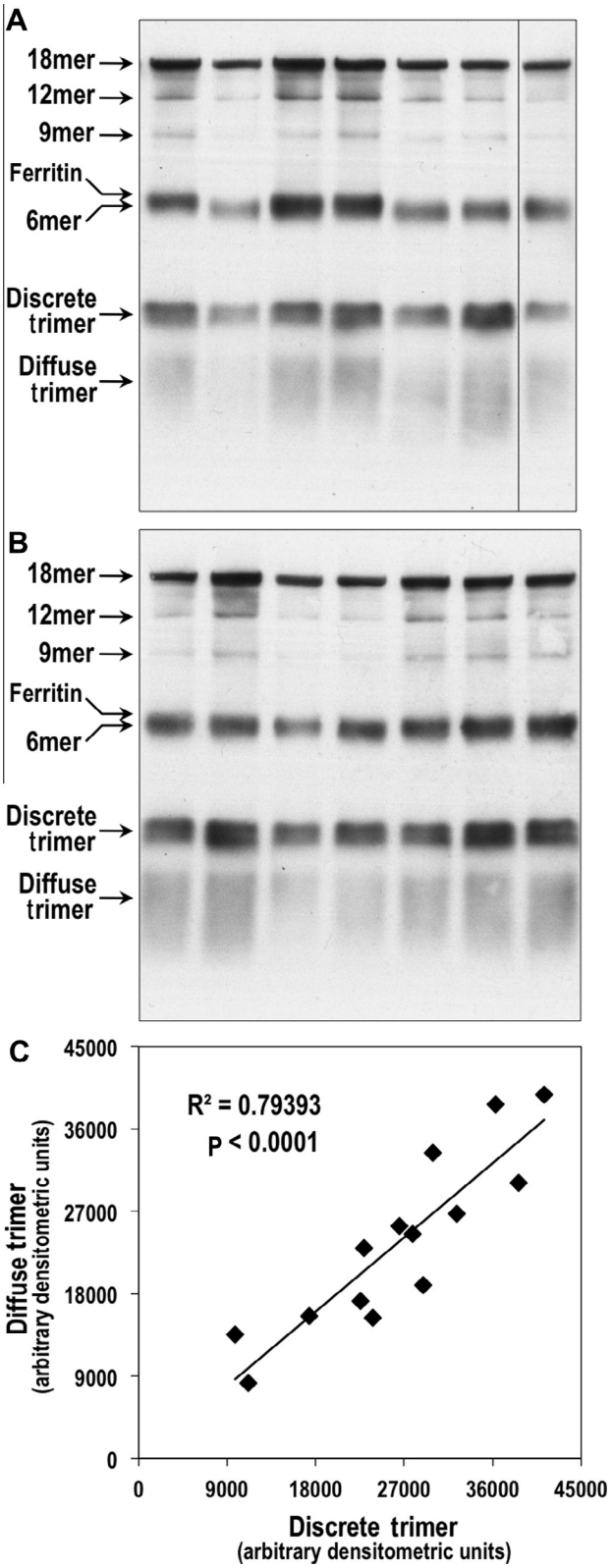

Fig.6. Native immunoblot analysis of human adiponectin oligomers in female (A) and male (B) control subjects. Human plasma (1 μl) diluted in native sample loading buffer was fractionated in 7% Tris–acetate native PAGE followed by Western blotting using rabbit antiserum raised against recombinant globular adiponectin. Densitometry was performed using ImageJ software. Abundance of diffuse and discrete trimers within 1 subject is plotted against each other in panel C. Each diamond-shaped marker represents 1 subject. The R^2 value of linear regression and the P value of Pearson's correlation analysis of all subjects are shown within the plot.

samples in a time- and cost-efficient manner. We previously used 7% Tris–acetate native acrylamide gels to electrophoretically separate adiponectin oligomers purified from bovine serum to study the mechanisms of adiponectin oligomerization in vitro [21,48,49]. To test whether the same technique is suitable for separating adiponectin oligomers in circulation, we performed immunoblot analysis of human and mouse plasma and bovine and mouse serum using a polyclonal antibody raised against the globular domain of adiponectin following fractionation in 7% Tris–acetate gels. To assess oligomerization states of adiponectin species in serum or plasma by comparison, purified bovine adiponectin 18mers and 6mers previously validated using equilibrium sedimentation or mass spectrometry were loaded onto the same gel along with fractions of human plasma eluted from a 16/60 Superdex 200 size exclusion column. SEC fractions (Fxs) 49.0–49.6, 55.6–56.2, and 62.8–63.4 ml were previously shown to correspond to HMW, 6mer, and trimer mouse adiponectin peaks, respectively [7]. The largest adiponectin species in all plasma or serum samples is 18mer because their migration distances were almost identical to that of purified bovine 18mers (Fig. 1A), which was confirmed by mass spectrometry. Major bands corresponding to purified bovine 6mers were observed in all plasma or serum samples (Fig. 1A). Two adiponectin species (A and B) with migration distances between 18mers and 6mers were clearly visible in human plasma and in SEC Fxs containing HMW adiponectin (Fig. 1A). Plotting migration distances against the natural logarithm of subunit numbers, species A and B most likely consist of 12 and 9 monomers, respectively, because those subunit numbers allow them to fall on a near straight line between 18mers and 6mers (Fig. 1B).

Bovine serum adiponectin at pH 7.0 and pH 5.0 as 18mer and 6mer standards

The predominant adiponectin species in bovine or fetal bovine serum was shown to be the 18mers (Fig. 1A). This suggests that bovine or fetal bovine serum could be used as adiponectin 18mer standard in native immunoblot analysis because there should be no ambiguity in assigning its oligomer state. However, a different oligomer standard is also needed as a second point of reference. Treating purified bovine adiponectin 18mers with a glycine-based buffer to lower the pH to 4.0 was previously shown to convert 18mers to 6mers [21]. Therefore, we assessed whether the same treatment would convert the 18mers in fetal bovine serum to 6mers. As shown in Fig. 2A, lowering the pH of fetal bovine serum or purified fetal bovine adiponectin solution to 4.0 led to conversion of 18mers to two smaller species. To assess the oligomerization state of purified adiponectin after a decrease in pH, we performed native nano-electrospray ionization mass spectrometry. This procedure confirmed that purified bovine adiponectin is predominantly 18mers (Fig. 2B, top panel). The major species after pH 4.0 treatment was shown to be 6mers, whereas a minor 12mer species was also observed (Fig. 2B, lower panel). These results indicate that fetal bovine serum at pHs 7.0 and 4.0 can serve as 18mer and 6mer standards in native immunoblot analysis. Ideally, a third oligomer standard will make assignment of adiponectin oligomers in biological samples even more accurate. Unfortunately, although a combination of low pH and reducing agent treatment can convert purified 18mers to trimers, the same treatment led to precipitation of proteins, including adiponectin, in fetal bovine serum.

Presence of two distinct adiponectin trimers in human and mouse plasma

Human and mouse plasma contained two adiponectin oligomers, a discrete and sharp species C and a diffuse species D, that

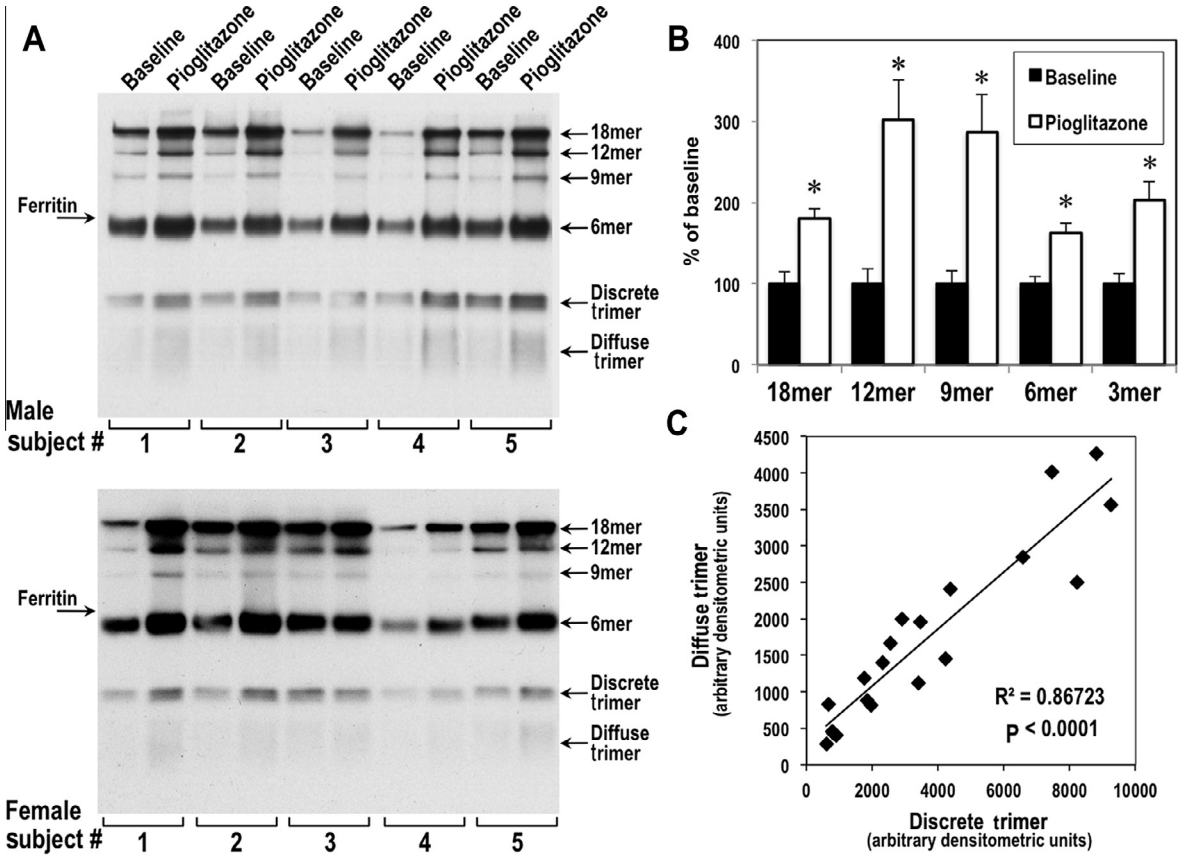

Fig.7. Native immunoblot analysis of adiponectin oligomers in human plasma from type 2 diabetic patients before and after 12 weeks of pioglitazone intervention. (A) Plasma (1 μl) from 5 male and 5 female patients each before and after 12 weeks on pioglitazone was fractionated in 7% Tris–acetate native PAGE followed by Western blotting using rabbit antiserum raised against recombinant globular adiponectin. Densitometry was performed using ImageJ software. (B) Fold increase in each oligomer after pioglitazone intervention. Abundance of each oligomer averaged from all 10 patients before pioglitazone treatment was used to normalize the amount of that oligomer in individual patients both before and after pioglitazone. For this analysis, levels of discrete and diffuse trimers were added to yield a total trimer level for each individual. (C) Correlation analysis of discrete and diffuse trimer abundance in plasma before and after pioglitazone intervention. Abundance of diffuse and discrete trimers within 1 subject was plotted against each other. The R^2 value of linear regression and the P value of Pearson's correlation analysis of all subjects are shown within the plot.

migrated faster than 6mers (Fig. 1A). To determine the composition of these two oligomers, human plasma was fractionated by SEC and the distributions of adiponectin oligomers in the fractions were assessed using both native (Fig. 3A) and denaturing (Fig. 3B) immunoblot analyses. The same analysis was also performed on rat plasma (Fig. 3C). Species D is most likely a trimer because it eluted from 16/60 Superdex 200 size exclusion column at the same position as purified recombinant adiponectin trimer (Fig. 3A and C and Ref. [7]). Species C eluted from the column after 6mers but before the diffuse species D (Fig. 3A and C). Adiponectin oligomers composed of four or five monomers are unlikely to form in native conditions because the X-ray crystal structure of the globular domain indicates that a tight hydrophobic core is created by three interfacing monomeric subunits [15]. Species C could be a hyper-sialylated, and therefore more negatively charged, LMW species with a more compact conformation. Such a species would elute later by SEC and migrate further by native gel electrophoresis than a species with less sialylation. To test this possibility, we treated human plasma and fractions of human plasma from size exclusion columns with sialidase. Following removal of negatively charged sialic acids, all adiponectin oligomers in human plasma migrated less than the analogous oligomer in the untreated sample (Fig. 4). On sialidase treatment, an oligomer whose migration distance deviated from untreated species C with a magnitude and direction similar to other adiponectin oligomers was observed (Fig. 4). This indicates that species C is not hyper-sialylated 6mer but rather a trimer. Species C may be a trimer bound to another protein in circulation. Consistent with this interpretation, species C is found in mouse serum but not in conditioned media from 3T3-L1 adipocytes cultured in the absence of serum or bovine serum albumin (Figs. 1A and 5A).

To further assess whether species D represents the trimeric form of human adiponectin in circulation, purified bovine 18mers were converted to 6mers and trimers and fractionated in native gels alongside human plasma. As shown in Fig. 5A, all of the bovine oligomers migrated farther down the gel than the human oligomers, which is consistent with the theoretical isoelectric point of bovine adiponectin relative to human adiponectin (5.34 vs. 5.46, calculated based on primary sequence without signal peptide using the ProtParam tool on the Swiss Bioinformatics Institute's ExPASy website). Bovine trimers are diffuse bands, as is species D (Fig. 5A). Plotting the migration distances of bovine 18mers, 12mers, 9mers, 6mers, and trimers against the natural logarithm of subunit number yielded a curve that was fitted best using a logarithmic regression model (Fig. 5B). Using a similar logarithmic regression model to fit the data points corresponding to human adiponectin oligomers, species D had a closer fit than species C as trimer (Fig. 5B). This indicates that species D, the diffuse trimer, represents the trimeric form of adiponectin not bound to other proteins.

Close correlation of circulating levels of discrete and diffuse trimers

To determine whether distribution of the discrete trimer, species C, is independent of the diffuse trimer, species D, we examined

the plasma levels of both types of trimers in a cohort of normal female (Fig. 6A) and male (Fig. 6B) individuals using 7% native Tris–acetate gels. The relative levels of discrete trimer and diffuse trimer from the same individual were plotted against each other, as shown in Fig. 6C. This analysis showed that the plasma levels of the two types of trimers are closely associated with each other over a 4-fold difference in adiponectin levels.

Effect of pioglitazone on plasma levels of adiponectin oligomers

The TZD class of insulin-sensitizing drugs is known to increase circulating adiponectin levels [11], with many studies showing proportionately higher increases in HMW adiponectin [12]. To assess the effect of a TZD drug, pioglitazone, on individual adiponectin oligomers, plasma from 5 male and 5 female diabetic patients randomly selected from a larger study [52] before and after 12 weeks of pioglitazone intervention was fractionated in 7% Tris–acetate gels under native conditions, and levels of individual adiponectin oligomers were determined by immunoblotting (Fig. 7A). Levels of all oligomers increased following pioglitazone intervention, with the largest fold increases being in 9mers and 12mers (Fig. 7B). Levels of discrete and diffuse trimers remained strongly associated with each other either at baseline or after 12 weeks on pioglitazone (Fig. 7C).

Effect of protein concentration on adiponectin oligomer assembly in vitro

The mechanisms by which TZDs selectively increase circulating HMW adiponectin remain unclear [12,53]. To determine whether increased total intracellular adiponectin concentration inherently leads to preferential production of HMW adiponectin, we examined the relationship between protein concentration and assembly of various adiponectin oligomers in vitro. Using reduced adiponectin trimers prepared from purified bovine 18mers as starting substrate, oxidative reassembly of adiponectin 6mers, 9mers, 12mers, and 18mers was initiated using hydrogen peroxide as an electron acceptor, and the reaction products were assessed using native and nonreducing denaturing PAGE (Fig. 8A and B). With doubling and quadrupling in the trimer concentration at the start of reassembly reactions, the amounts of 6mers assembled increased by 2.0 ± 0.3-fold and 3.8 ± 0.6-fold accordingly (Fig. 8C). Without differences in the extent of total adiponectin oxidation (Fig. 8B), the amounts of 18mers assembled increased disproportionately compared with those of 6mers, with each doubling in starting trimer concentration (Fig. 8A and C). There was a tendency for increased assembly of 9mers and 12mers (Fig. 8A and C), but this was not statistically significant compared with the amount of 6mers assembled. These results suggest that increased adiponectin production could lead to preferential enhancement of HMW adiponectin formation, but the pattern of increase in the three individual HMW oligomers was different from that in human plasma after 12 weeks of pioglitazone intervention (Figs. 7 and 8). No discrete trimer (species C) was observed in adiponectin oligomerization assembly in vitro, which further supports the notion that species C is a trimer bound to another yet unidentified serum protein.

Discussion

Several analytical techniques have been previously applied to determine the oligomerization states of recombinant adiponectin or adiponectin in serum or plasma. These include velocity sedimentation in discontinuous sucrose density gradient [32,36], SEC [18,35], nonreducing and nonheating SDS–PAGE [19,37,38,54–56], and oligomer-specific ELISA [45–47]. All of these techniques

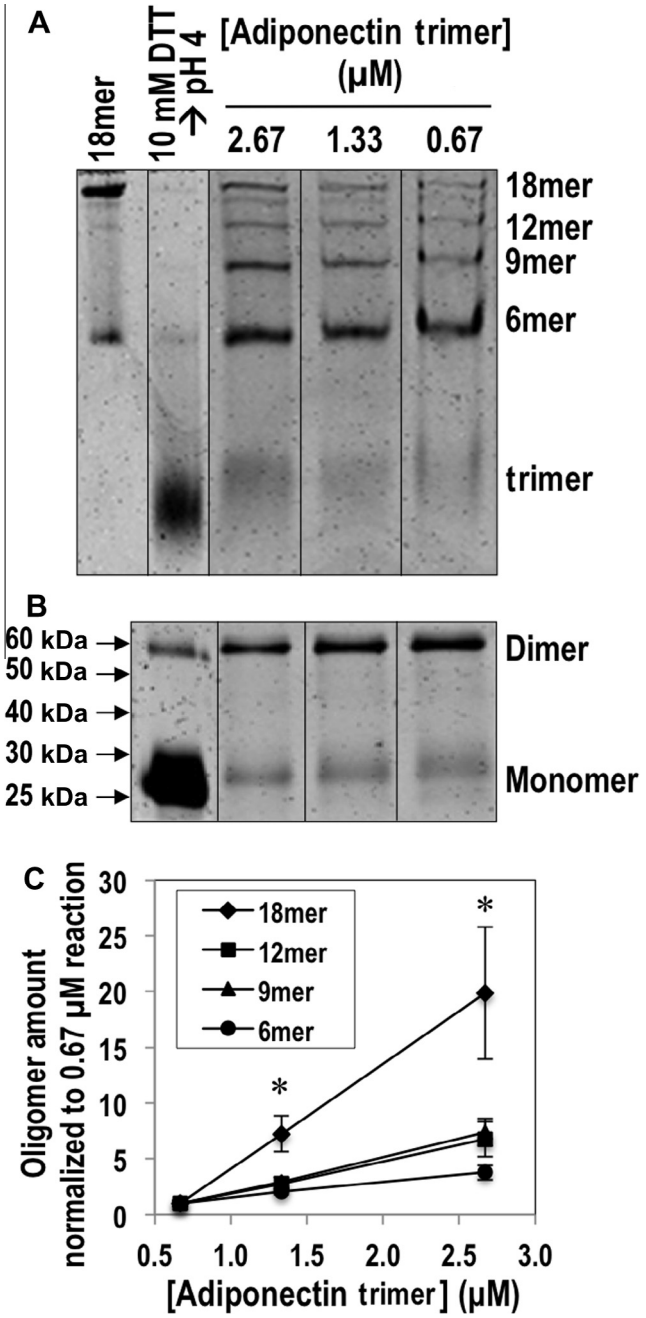

Fig.8. Effect of starting trimer concentration on adiponectin reoligomerization. Purified HMW adiponectin was collapsed to trimers by sequential treatment in 10 mM DTT and 50 mM glycine (pH 4.0) for 30 min at 37 °C each and then dialyzed against PBS in an anaerobic chamber for at least 3 h to remove DTT and to return pH to 7.0. The samples were diluted to 2.67-, 1.33-, and 0.67-μM trimers using PBS. (A and B) Adiponectin oxidation and reoligomerization were initiated by 3 mM H_2O_2 and allowed to proceed for 45 min at 25 °C before the reactions were analyzed using native PAGE analysis for oligomer distribution (A) and nonreducing SDS–PAGE for redox states (B). Although concentrations of trimers differed in each reaction, same amounts of adiponectin were loaded unto native and denaturing gels to ease comparison among samples. Gels were stained with Coomassie Brilliant Blue G-250, and band intensities were quantified after scanning using infrared fluorescence imaging as described in Materials and Methods. (C) Relative concentration of 18mers, 12mer, 9mers, and 6mers formed during reassembly reactions plotted against starting trimer concentration. Densitometric measurements of bands corresponding to individual oligomers are normalized to both the volumes of reactions loaded and the amounts of each oligomer formed in the reactions with the lowest concentration of starting trimers. Results are averaged from three independent experiments. An asterisk (∗) denotes $P < 0.05$ against the amount of 6mers formed at a particular trimer concentration during reassembly reactions in one-way ANOVA with Tukey's HSD post hoc test.

have certain disadvantages. Although sucrose gradients allow facile separation of HMW and hexameric adiponectin, their resolution in the LMW range is poor. SEC is a low-throughput technique. Nonreducing and nonheating SDS–PAGE using gradient gels (2–15% in Ref. [19], 4–20% in Ref. [54], and 3–8% in Refs. [37], [38], and [55]) provides excellent separation of adiponectin oligomers. However, this procedure could potentially yield monomeric or dimeric adiponectin if not carefully performed to avoid conditions in which heat is not needed to accelerate the denaturing effects of SDS. These conditions include higher concentrations of SDS and/or prolonged exposure to SDS. Thus, it is necessary to develop a simple technique that could accurately and rapidly assess adiponectin oligomerization states.

Native gel electrophoresis in which SDS or other denaturing agents are completely absent in both the sample and the running buffers has been used previously to assess adiponectin oligomerization state in serum [39,41,42,57]. Compared with the 5% Tris–glycine [39] and the gradient gel-based systems [41,42,57], the 7% Tris–acetate gel electrophoresis used in this study has certain advantages. In the Tris–acetate electrophoresis system, acetate and glycinate ions serve as leading and trailing ions, respectively. Compared with the fast-moving chloride ions in the Tris–HCl gels commonly used in Tris–glycine electrophoresis systems, the slower moving acetate (due to the relatively high pK_a of acetic acid) leads to a lower voltage gradient between the leading and trailing ions to facilitate stacking and migration of large protein complexes such as adiponectin oligomers. The usefulness of acetate was also shown in a study by Peake and coworkers in which excellent separation of human adiponectin oligomers was achieved in Tris–glycine gels following incubation of samples in 0.1 M acetate [44]. Recently, a blue native PAGE system using Coomassie Brilliant Blue G-250 has been successfully used to separate different oligomeric forms of adiponectin in serum [41,42] or human breast milk [58] using commercially available precast gradient gels. It is significantly easier to cast 7% Tris–acetate gels using published procedures [21] than to cast gradient gels. Because adiponectin is negatively charged under native conditions, it is not necessary to use Coomassie dye to confer negative charge during electrophoresis.

A potential disadvantage associated with native gel-based immunoblot analysis of adiponectin oligomers may occur if the large and small oligomers differ in transfer efficiency. Larger proteins such as 18mers might not transfer as efficiently as 6mers or trimers; therefore, depending on completeness of the transfer process, the relative abundance of each oligomer could be skewed. Although we were not able to directly assess the transfer efficiency of serum adiponectin oligomers from gel to membrane due to the difficulty of assessing the amount of adiponectin remaining in gel, we found that the ferritin (up to at least 25 μg) and the PageRuler prestained molecular weight marker (up to at least 10 μl) transferred 100% by visual inspection of the gels (data not shown). However, purified adiponectin 18mers up to 6.75 μg per lane did not fully transfer. Approximately 25% of the purified 18mers remained in gels untransferred to the membrane routinely (data not shown). Regardless, comparative abundance of a particular oligomer within a series of samples is independent of transfer efficiency.

In nano-electrospray ionization mass spectrometry, the 18mers purified from bovine serum presented as one major and one minor charge distribution (Fig. 2B). Similarly, at pH 4.0, the hexamers also presented as two distinct charge distributions (Fig. 2C). Charge states of proteins in electrospray have been shown to correlate with their surface area, and glycosylation can affect charging of proteins [59]. Proteins sprayed in aqueous buffers under gentle conditions usually exhibit a single distribution of charge states, implying a single folded form. The bimodal charge state distributions of the adiponectin oligomers indicate that there might be multiple forms of each type of oligomer. It is likely that the two distributions are differentially glycosylated. Another potential explanation is differential binding to metals. Adiponectin is known to bind Zn(II) and Ca(II) ions [35,49,54], and differences in the amount of bound ions could lead to different charge state distributions. However, the resolution is not sufficient to confidently distinguish small mass differences on these oligomers; thus, the molecular bases responsible for the bimodal charge state distributions are currently unclear.

Whether individual adiponectin oligomers undergo interconversion following secretion from cells has important functional implications in adiponectin action. With the possible exception of elastase-mediated generation of globular adiponectin [60], several independent studies, each using a different experimental approach, have uncovered no evidence of oligomer interconversion during circulation [32,35,39]. However, because none of these studies employed analytical procedures capable of separating 18mers from 12mers and 9mers, whether these HMW oligomers interconvert during circulation was not addressed. The fact that it is possible to separate 9mers, 12mers, and 18mers in native gels indicates that if there is exchange among these oligomers, the rate must be slow compared with the speed of migration through gel. Similarly, the abundance of 9mers in gel filtration column fractions was clearly distinguishable from those of 12mers and 18mers, indicating the absence of fast oligomer interconversion involving 9mers (Fig. 2). The resolution of the Superdex 200 16/60 column was not sufficient to determine whether 18mers and 12mers had distinct elution profiles (Fig. 3). Using quaternary ammonium ion exchange chromatography, 9mers, 12mers, and 18mers also had distinct elution profiles (data not shown), further suggesting slow or lack of exchange among these three HMW adiponectin species. The observation that pioglitazone treatment led to a greater increase in circulating 9mers and 12mers compared with 18mers also suggests that these oligomers do not readily interconvert in circulation (Fig. 7).

A major finding uncovered by the native PAGE technique is the presence of two distinct trimeric adiponectin species in both human and mouse plasma: a sharp, slow-migrating discrete species (C) and a diffuse, fast-migrating species (D) (Fig. 1A). The diffuse nature of species D in native gel suggests that it has a high degree of heterogeneity in conformation or extent of glycosylation. Adiponectin is glycosylated extensively [61], and the sialic acid content in adiponectin is associated with its circulating half-life [62]. Despite a diffuse appearance in native gels, this trimer was visible over a similar number of fractions in gel filtration chromatography as other oligomers that appeared to be much more compact on native gels, suggesting that the diffuseness was caused by heterogeneity in protein charge rather than conformation. This interpretation is consistent with conversion of the diffuse trimer to a sharper and slower moving species following sialidase treatment (Fig. 4).

The absence of discrete trimer (species C) in conditioned media from 3T3-L1 adipocytes (Fig. 1A) and the elution profile from SEC (Fig. 3) suggest that species C is a complex formed between adiponectin trimer and an unknown plasma protein. A prior study reported that a portion of adiponectin in human plasma is tightly bound to albumin [57]. The tight association between the abundance of the discrete and diffuse trimers (Figs. 6C and 7C) suggests a simple bimolecular association–dissociation equilibrium between adiponectin trimer and the putative interacting protein and also that the interacting protein is not limiting.

The ability of TZDs to increase circulating adiponectin is well documented [10]. However, there is disagreement regarding whether HMW adiponectin is better than total adiponectin in assessing the efficacy of TZD treatment [63]. Results in the current study indicate that in a limited cohort of diabetic patients,

pioglitazone intervention led to increased levels of all adiponectin oligomers, with the largest fold increases being observed with 9mers and 12mers (Fig. 7). Commercially available assays for measuring HMW adiponectin vary considerably in methodology, and currently there is no information regarding whether these assays detect 18mers, 12mers, and 9mers with equal efficiency. Considering that pioglitazone treatment led to preferential increases in 9mer and 12mer levels, we speculate that potential assay variability in detecting the three distinct HMW adiponectin species could lead to different interpretations of the effect of TZD treatment on HMW adiponectin levels.

Insulin-sensitizing actions of adiponectin have been described for the HMW and the trimeric/globular isoforms [12,64–66] but with relatively lower or lack of activity attributed to the hexamers [13,16,35]. The molecular mechanisms underlying this phenomenon remain unclear at this time. It has been reported that the AdipoR1 receptor has higher affinity for globular adiponectin, whereas the AdipoR2 receptor has higher affinity for full-length adiponectin [67]. It is possible that the AdipoR1 receptor has more robust activity when bound singly to trimeric forms of adiponectin (with globular adiponectin being exclusively trimeric), whereas the AdipoR2 receptor achieves maximal activity through receptor clustering by interacting with multiple trimeric subunits held together in the form of HMW adiponectin. If this model is true, the predicted activity of 9mers and 12mers in enhancing insulin sensitivity is expected to be higher than hexamers but lower than 18mers.

We have previously used an in vitro approach to explore the basic biochemical principles that govern assembly of adiponectin oligomers [21,48,49]. Here we took the same approach to address whether the TZD-mediated increase in adiponectin concentration in serum inherently leads to enhanced production of HMW adiponectin. Our results show that an increased starting adiponectin trimer concentration led to a disproportionate increase in the amount of 18mer assembled (Fig. 8). We propose that HMW adiponectin assembly is dependent on concentration of trimers. The mechanism is likely a concentration-dependent increase in the rate of inter-trimer disulfide bond formation relative to that of intra-trimer disulfide bonding, which is concentration independent. Whereas inter-trimer bonding facilitates oligomerization, intra-trimer bonding precludes further oligomer assembly [48]. This finding could explain the tight association between total and HMW adiponectin levels in many studies [68] and the preferential correlation between HMW adiponectin and improved metabolic outcome following TZD treatment [5]. However, additional factors must be involved in the regulation of HMW and total adiponectin blood concentration given that pioglitazone intervention was associated with selective increases in 9mers and 12mers rather than 18mers (Fig. 7).

In summary, the combined use of 7% Tris–acetate native PAGE and methods for producing adiponectin 18mer and 6mer standards allows for clear identification of six different oligomers in human plasma: 18mer, 12mer, 9mer, 6mer, discrete trimer, and diffuse trimer. Biochemical analysis of individual oligomers following pioglitazone intervention and the oligomer assembly process indicates that total adiponectin concentration is a crucial factor in enhanced production of HMW adiponectin. Future work focusing on individual adiponectin oligomers rather than HMW adiponectin as a whole may reconcile some of the discrepancies in the current literature regarding adiponectin action and regulation of its production.

Acknowledgments

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