Adiponectin Isoform Patterns in Ethnic-Specific ADIPOQ Mutation Carriers: The IRAS Family Study

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Objective: Adiponectin is found in human serum in three groups of multimers (high molecular weight [HMW], medium molecular weight [MMW], and low molecular weight [LMW]). Two ethnic-specific variants in ADIPOQ, G45R (Hispanic-Americans) and R55C (African-Americans), were previously reported. Although carriers of both variants had mean adiponectin levels ≤20% of those of noncarriers, they were not clinically different from noncarriers. To compare carriers of both variants and noncarriers, relative quantification of adiponectin isoforms to total adiponectin was performed on serum samples.

Methods: The multimeric patterns of serum adiponectin in G45R carriers (n = 23), R55C carriers (n = 3), and Hispanic- and African-American noncarriers (n = 84 and 44, respectively) from the Insulin Resistance Atherosclerosis Family Study were explored using native Western blotting and densitometry.

Results: Serum samples from carriers showed an absence of the HMW isoform and a marked reduction in the MMW isoform but an approximate twofold increase in the amount of the LMW isoform. Thus, individuals making only LMW adiponectin are metabolically normal.

Conclusions: The results contrast with the proposed biological importance of the HMW multimer. This suggests that the LMW isoform may functionally compensate for some of the loss or reduction of the higher-order multimers in carriers of the G45R and R55C mutations.

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Introduction

Adiponectin, encoded by the ADIPOQ gene, is an adipokine exclusively synthesized and secreted by adipocytes into serum (1). Levels of circulating adiponectin have been shown to be inversely correlated with body weight and cardiometabolic disease (2-5) and have been the subject of extensive investigation in metabolic disease in humans. The adiponectin monomer contains a collagen-like domain that facilitates assembly of multimers containing carboxyl terminal globular domains believed to be the active component of the molecule. In normal human serum, adiponectin is distributed in three distinct isoforms: low-molecular-weight (LMW) trimers, medium-molecular-weight (MMW) hexamers, and high-molecular-weight (HMW) oligomers (1,6). The HMW oligomer, composed of 12 to 18 adiponectin monomers, has previously been reported to be the most biologically active of the three isoforms in both humans and mice (7,8), although there is not a firm consensus on this position in the literature. In addition, there is evidence that the ratio of HMW to total adiponectin is a better predictor of insulin resistance and metabolic syndrome when compared with total adiponectin levels alone (6,9).

Previously, we have reported identification of two ethnic-specific novel coding variants in the ADIPOQ gene: G45R in Hispanic-Americans (10) and R55C in African-Americans (11). These coding variants are located in the collagen-like domain of ADIPOQ. Total serum adiponectin levels had previously been quantified by radioimmunoaassay (RIA) (12) in individuals harboring the variants and were found to be ≤20% of the mean adiponectin levels observed in noncarriers (10). Despite the documented inverse correlation

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between cardiometabolic disease and total serum adiponectin levels (2), these G45R and R55C mutation carriers were statistically no different in measures of metabolic health (e.g., insulin sensitivity, BMI) and were not clinically different from related individuals who do not harbor the G45R or R55C variants (10,11). Based on the seemingly contradictory results observed in individuals with mutations, we have carried out a focused analysis of the adiponectin isoforms from these subjects in an effort to explain the surprising lack of association with clinical characteristics commonly associated with a reduction in serum adiponectin levels.

Methods

Materials

Primary rabbit polyclonal antibodies against mouse and human adiponectin were obtained from BioVendor R&D Products (Asheville, North Carolina). Fluorescently labeled 800CW Goat anti-Rabbit immunoglobulin G secondary antibody, nitrocellulose membranes, and Odyssey Tris-buffered saline blocking buffer were purchased from LI-COR Biotechnology (Lincoln, Nebraska). Reference HMW adiponectin isoforms were purchased from R&D Systems (Minneapolis, Minnesota). Control pooled mouse serum and the Life Technologies total adiponectin antibody was then added to each well, followed by horseradish peroxidase solution. Finally, tetramethylbenzidine substrate was used to detect horseradish peroxidase activity. The addition of stop solution changed the reaction color for detection at 450 nm by a microplate reader.

Western blots

Native (nondenaturing, nonreducing) Western blots were performed to separate and visualize the adiponectin isoforms within each serum sample (human and mouse). Each 20-µL sample consisted of 0.5 µL of serum, 10 µL of standard native loading buffer, and 9.5 µL of 50 mM Tris buffer, pH 6.8. Samples were loaded onto hand-cast 6% polyacrylamide gels in Tris-glycine buffer without sodium dodecyl sulfate and run for 3 hours and 30 minutes at 125 V on ice. Following electrophoresis, gels were soaked for 15 minutes in 0.1% sodium dodecyl sulfate to confer a net negative charge to the separated proteins and facilitate the unidirectional transfer to a nitrocellulose membrane. Protein transfer was conducted in Tris-glycine buffer with 20% methanol at 25 V for 1 hour. After transfer, the membranes were fixed in 5% acetic acid for 15 minutes, rinsed with deionized water, and allowed to air-dry for several minutes. Adiponectin was probed using a 1:2,500 dilution of a species-specific polyclonal rabbit anti-adiponectin antibody. A fluorescently labeled secondary antibody (IRDye 800CW Goat anti-Rabbit immunoglobulin G) was used to detect the adiponectin-bound primary antibody. Following washing to remove the excess unbound antibody, the membranes were dried for 24 hours and scanned using an Odyssey Classic Infrared Imaging System (LI-COR Biotechnology). Scans were saved in a high-resolution image format for subsequent analysis.

Densitometry analysis and quantification

Fiji ImageJ software (16,17) was used to quantify the adiponectin isoforms for each sample. Each image was separated out by color channel (red, blue, and green). Subsequent steps were performed using the green channel image. Three regions corresponding to the HMW, MMW, and LMW bands of adiponectin were delineated. Rectangles were drawn around each lane of the gel, and the total intensity of the signal within each designated isoform region was measured. The signal score was calculated by subtracting the background signal from the total intensity. The average intensity of the marker lanes from the red channel image was used as the reference signal, and each signal score was divided by the reference signal to obtain the final adjusted signal score, or relative intensity value. Densitometry traces were graphed based on the relative intensity values with Microsoft Excel, and total adiponectin was estimated for each sample by summing the relative intensities for that sample. Total adiponectin levels and isoform to total adiponectin percentages were determined based on these values.

Results

Table 1 lists relevant demographic information for the IRASFS participants whose sera were tested in this study. Among the noncarrier samples were Hispanic-American and African-American individuals who previously had low (bottom 20%) total plasma adiponectin levels by RIA (12). These samples were chosen for analysis to investigate the possibility that they also showed isoform distribution differences such as those observed with the carrier samples. In addition, among Hispanic-Americans only, noncarriers
| Traits                          | Hispanic-Americans | African-Americans |
|--------------------------------|---------------------|-------------------|
|                                | Noncarriers, standard | Noncarriers, low | G45R carriers | Noncarriers, low | R55C carriers |
| n                              | Mean | Range | Mean | Range | Mean | Range | Mean | Range | Mean | Range |
| Age (y)                        | 43.37 | 20-73 | 39.06 | 19-76 | 41.28 | 21-70 | 41.32 | 20-68 | 45.51 | 33-56 |
| % Female                       | 61.5 | 21.4 | 56.5 | 20.7 | 13 | 66.7 | 45.5 | 66.7 | 66.7 |
| % T2D                          | 7.7 | 20.7 | 2.20 | 1.20-3.00 | 2.87 | 1.20-3.67 | 1.34 | 1.20-1.54 |
| Adiponectin (μg/mL)            | 13.84 | 7.24-20.22 | 13.84 | 7.24-20.22 | 13.84 | 7.24-20.22 | 13.84 | 7.24-20.22 |
| Glucose homeostasis            | 1.64 | 0.13-5.01 | 1.64 | 0.13-5.01 | 1.64 | 0.13-5.01 | 1.64 | 0.13-5.01 |
| AIR (pmol/L)                   | 1,188.78 | 338.45-3,900.30 | 1,188.78 | 338.45-3,900.30 | 1,188.78 | 338.45-3,900.30 | 1,188.78 | 338.45-3,900.30 |
| DI (SI × AIR × 10^{-5} min^{-1}) | 1,561.08 | 101.01-5,988.99 | 1,561.08 | 101.01-5,988.99 | 1,561.08 | 101.01-5,988.99 | 1,561.08 | 101.01-5,988.99 |
| Fasting glucose (mg/dL)        | 95.86 | 80.5-125 | 95.86 | 80.5-125 | 95.86 | 80.5-125 | 95.86 | 80.5-125 |
| Fasting insulin (μU/mL)        | 16.20 | 13-221.5 | 16.20 | 13-221.5 | 16.20 | 13-221.5 | 16.20 | 13-221.5 |
| Metabolic clearance of insulin (min^{-1}) | 18.95 | 13-221.5 | 18.95 | 13-221.5 | 18.95 | 13-221.5 | 18.95 | 13-221.5 |
| Adiposity                      | 30.2 | 17.8-37.5 | 30.2 | 17.8-37.5 | 30.2 | 17.8-37.5 | 30.2 | 17.8-37.5 |
| BMI (kg/m²)                    | 124.36 | 37.16-220.93 | 124.36 | 37.16-220.93 | 124.36 | 37.16-220.93 | 124.36 | 37.16-220.93 |
| Subcutaneous adipose tissue (cm²) | 400.34 | 67.21-668.19 | 400.34 | 67.21-668.19 | 400.34 | 67.21-668.19 | 400.34 | 67.21-668.19 |
| Waist circumference (cm)       | 93.25 | 71.75-112.90 | 93.25 | 71.75-112.90 | 93.25 | 71.75-112.90 | 93.25 | 71.75-112.90 |
| Liver density (HUL)            | 52.94 | 25.71-66.53 | 52.94 | 25.71-66.53 | 52.94 | 25.71-66.53 | 52.94 | 25.71-66.53 |
| Triglycerides (mg/dL)          | 116.12 | 24-234 | 116.12 | 24-234 | 116.12 | 24-234 | 116.12 | 24-234 |
| High-density lipoproteins (mg/dL) | 39.92 | 13-95 | 39.92 | 13-95 | 39.92 | 13-95 | 39.92 | 13-95 |
| Low-density lipoproteins (mg/dL) | 108.08 | 34-177 | 108.08 | 34-177 | 108.08 | 34-177 | 108.08 | 34-177 |
| Total cholesterol (mg/dL)      | 171.23 | 102-246 | 171.23 | 102-246 | 171.23 | 102-246 | 171.23 | 102-246 |
| Systolic blood pressure (mmHg) | 119.27 | 98-197 | 119.27 | 98-197 | 119.27 | 98-197 | 119.27 | 98-197 |
| Diastolic blood pressure (mmHg) | 79.46 | 64-99 | 79.46 | 64-99 | 79.46 | 64-99 | 79.46 | 64-99 |
| Albumin creatinine ratio (g/g) | 16.25 | 3.65-109.44 | 16.25 | 3.65-109.44 | 16.25 | 3.65-109.44 | 16.25 | 3.65-109.44 |
| Inflammation                   | 6.26 | 0.13-32.81 | 6.26 | 0.13-32.81 | 6.26 | 0.13-32.81 | 6.26 | 0.13-32.81 |

aDue to sample availability, all IRASFS African-American samples included in this study were either noncarriers measured to have low adiponectin levels by RIA or R55C carriers.

b"Standard" refers to those samples with total adiponectin levels measured to fall within the mean ± 1 standard deviation of all samples measured; those samples in the bottom 10% of all samples measured were classified as "low" (12).

cAdiponectin levels as measured by RIA (12).

Abbreviations: AIR, acute insulin response; DI, disposition index; SI, sensitivity index; RIA, radioimmunoassay.
with normal and/or average plasma adiponectin levels were included. Thus, there were three Hispanic-American test groups and two African-American test groups. Across most measures (e.g., BMI), the sample subgroups were not noticeably different. For some traits, these small samples differed (e.g., acute insulin response; Table 1). The ranges of acute insulin response measurements were large; however, the median values showed more modest differences (e.g., Hispanic-American, normal adiponectin: 810.80 pmol/L; Hispanic-American, low adiponectin: 625.50 pmol/L; Hispanic-American, G45R carriers: 497.80 pmol/L). Moreover, in the original analyses (10,11), there were no significant differences based on genotype found in the overall samples (Hispanic-American, n = 1,240; African-American, n = 566).

**ELISA**
Serum adiponectin levels of the 26 Hispanic-American samples from the IRASFS measured by ELISA ranged from 1.51 to 29.37 ng/mL (mean = 9.34 ng/mL; SD = 6.54). Plasma adiponectin levels had previously been measured in these IRASFS participants using RIA (12). This assay was followed by measurement on a subset of samples (n = 55) using a total adiponectin ELISA (EMD Millipore, Billerica, Massachusetts) (10). Among the three quantification assays, the highest correlation was between the results of the two ELISAs (r = 0.91). There was relatively poor correlation (r = 0.56) between the measurements obtained by the prior RIA and ELISA performed in this study, although this could be due in part to the use of serum compared with plasma as the source of adiponectin.

**Western blots**
Fluorescent imaging of Western blots showed that HMW isoforms were undetectable or greatly reduced and the MMW isoform was markedly reduced in G45R and R55C carrier samples (Figure 1A-1B, respectively). This was accompanied by a noticeable increase in the amount of LMW isoform in samples from both G45R and R55C carriers compared with noncarrier samples. In contrast to the human samples, fluorescent images of mouse serum immunoblots showed only the HMW and MMW isoforms of adiponectin (Figure 2), consistent with previously published results (7). Mouse samples incubated with a human antibody and human samples incubated with a
mouse antibody did show cross-reactivity, although the LMW isoform was only faintly detected in human samples when probed with a mouse antibody (data not shown).

Densitometry and quantification
Representative densitometry traces illustrating the distribution of the three adiponectin isoforms are shown for a Hispanic-American G45R carrier (Figure 3A), a Hispanic-American noncarrier (Figure 3B), an African-American R55C carrier (Figure 3C), and an African-American noncarrier (Figure 3D). The same patterns were consistently observed among individual members of each respective group. The average percentage of HMW to total adiponectin was determined from the quantification of blots for each group (Figure 4). Interestingly, the Hispanic-American noncarriers, low (RIA) adiponectin level test group appeared to have a lower HMW percentage than the Hispanic-American noncarriers, normal adiponectin level test group. Mean percentages of HMW for each group were as follows: G45R carriers, 1.0% (0.3%-1.6%); G45 noncarriers, low (RIA) adiponectin levels, 8.7% (1.8%-15.4%); G45 noncarriers, normal adiponectin levels, 14.1% (5.6%-24.9%); R55C carriers, 2.6% (1.2%-6.1%); and low (RIA) R55 noncarriers, 6.1% (1.3%-20.5%). The mean percentages of MMW for each group were as follows: G45R carriers, 4.6% (0.96%-33.3%); G45 noncarriers, low (RIA) adiponectin levels, 17.9% (10.4%-30.2%); G45 noncarriers, normal adiponectin levels, 23.5% (2.8%-38.2%); R55C carriers, 3.4% (1.8%-6.1%); and low (RIA) R55 noncarriers, 18.3% (8.7%-32.5%). In addition, using densitometry, we estimated that the LMW isoform synthesis was 2.2-fold higher in the G45R carriers and 2.5-fold higher in the R55C carriers than in noncarriers.
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Discussion

In this study, we performed native Western blotting experiments with densitometry analysis of adiponectin in serum samples from Hispanic-American and African-American IRASFS participants. The motivation was to use Western blots to assess serum adiponectin from individuals harboring the G45R or R55C ADIPOQ variants. It is important to note that before either variant had been identified, carriers were determined to have low total adiponectin levels by RIA but were not clinically different from their family members with higher adiponectin levels (10-12). Herein, we show that the difference is not the total adiponectin concentration, but the pattern of adiponectin isoform distribution between carriers of the G45R and R55C variants and noncarriers from the same populations. Working back to the original reports, we conclude that variable isoform distributions were not significantly different in their associations with cardiometabolic traits. With this, secretion of the LMW isoform of adiponectin was substantially increased in G45R and R55C mutation carriers.

It is striking that the low total adiponectin levels in G45R and R55C carriers determined previously by RIA (12) were so different from the current Western blot analysis. However, a marked increase in the LMW isoform and considerably decreased HMW and MMW were observed in Western blot analysis of the mutant carriers. This observation substantially changes the context of the discussion of why individuals with the G45R or R55C variants do not differ clinically from noncarriers. The apparent increase in LMW adiponectin in carriers of these mutations, leading to higher levels of total adiponectin, may functionally compensate for the lack of the HMW isoform. This advantage is not observed in carriers of other ADIPOQ variants, e.g., G84R and G90S, who, despite producing normal levels of LMW and MMW isoforms, are unable to form the HMW multimers and overall have lower total adiponectin levels and a higher incidence of metabolic disease (18,19). This scenario, however, results in further questions. First, it has been reported that alterations to the HMW multimer are in some cases correlated to increases in insulin sensitivity (7,8), but carriers of ADIPOQ G45R or R55C variants lacking the HMW isoform do not differ phenotype from Hispanic-American or African-American noncarriers. Thus, characterization of the isoform distribution of the G45R and R55C carriers provides interesting insight into the metabolic action of adiponectin isoforms, suggesting that LMW multimers may be comparably active and suggesting a need for investigation into the role of different isoforms. It is notable that endogenous LMW adiponectin makes up a smaller proportion of the total adiponectin in mice (18), which was replicated using this experimental approach (data not shown). These dramatic differences suggest that the metabolic influences of adiponectin isoforms may be different in this model system compared with humans.

Based on the discrepancy between the total adiponectin levels measured by RIA versus those measured by other antibody-dependent assays (ELISA and Western blot), it is likely that some antibodies may be subject to preferential detection of one isoform over others. In this case, it appears that the RIA may have preferentially detected the HMW isoform of adiponectin, resulting in an inaccurate quantification of total adiponectin levels of those participants carrying the G45R mutation. It is therefore worth noting that caution must be taken when selecting an antibody-based quantification system to measure serum adiponectin, or any protein with multiple isoforms, to ensure the desired isoform(s) is detected. Here, a rabbit polyclonal anti-human adiponectin antibody was used to successfully detect adiponectin isoforms, but other polyclonal antibodies have been used with similar results. In addition, we used a monoclonal anti-human adiponectin antibody and observed nearly identical results (data not shown). This study underscores the importance of antibody validation for the intended purpose of an experiment or assay.

Conclusion

Analysis of naturally occurring mutations in ADIPOQ reveal insights into adiponectin biology, including a possible compensatory mechanism to explain the clinically normal phenotype in G45R and R55C carriers who lack the HMW isoform.

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