Enhanced adiponectin multimer ratio and skeletal muscle adiponectin receptor expression following exercise training and diet in older insulin-resistant adults

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O’Leary VB, Joret AE, Marchetti CM, Gonzalez F, Phillips SA, Ciaraldi TP, Kirwan JP. Enhanced adiponectin multimer ratio and skeletal muscle adiponectin receptor expression following exercise training and diet in older insulin-resistant adults. Am J Physiol Endocrinol Metab 293: E421–E427, 2007. First published May 8, 2007; doi:10.1152/ajpendo.00123.2007.—Circulating adiponectin is secreted primarily from adipocytes and circulates as oligomeric complexes that determine its activity and influence over lipid metabolism. This protein subsequently became known as adiponectin, adiponectin multimer distribution, and skeletal muscle adiponectin receptor expression in older obese adults diagnosed with impaired glucose tolerance (IGT). The study design

MORE THAN A DECADE AGO, a novel secretory protein was discovered (31) with homology to collagen, a complement factor subunit, and an obscure protein associated with animal hibernation. This protein subsequently became known as adiponectin (AdiopQ, ACDC, Acrp30, apM-1, APM1, GBP28). Adiponectin is secreted primarily from adipocytes and circulates almost exclusively as homomultimeric full-length glycoprotein complexes that determine its activity and influence over lipid and carbohydrate metabolism (28). Low plasma adiponectin levels have been implicated in the development of insulin resistance and associated disorders such as obesity (30), hyperlipidemia (11), and type 2 diabetes (19). Evidence linking adiponectin with insulin action stems from increased insulin sensitivity upon adiponectin administration, resulting in an increase in skeletal muscle glucose uptake and hepatic fatty acid oxidation (38), as well as reports that reduced adiponectin expression occurs in parallel with the onset of insulin resistance in obese humans, rodents, and monkeys (2, 15, 17).

Likewise, increased physical activity, weight loss, and/or caloric restriction have been shown to significantly reduce insulin resistance (14, 26, 36). However, inconsistent findings have emerged from studies investigating the effects of exercise on circulating adiponectin levels (1, 3, 6, 20, 23, 29). To date, the mechanism by which adiponectin acts as an insulin-sensitizing hormone remains to be fully clarified. Nevertheless, new insights highlight the role of the relative distribution of adiponectin multimers as a more precise determinant governing adiponectin’s defensive properties against metabolic disorders (9, 28, 35).

Adiponectin multimers mediate both joint and independent responses activating signal transduction pathways through two distinct integral membrane proteins, AdipoR1 and AdipoR2, leading to downstream events such as lipid oxidation and glucose uptake. AdipoR1 and AdipoR2 acting as receptors for globular and full-length adiponectin show abundant expression in skeletal muscle and liver, respectively (37). It has been shown (10, 34) that decreased expression of both AdipoR1 and AdipoR2 in skeletal muscle of insulin-resistant obese mice and patients with type 2 diabetes leads to reduced adiponectin sensitivity and insulin resistance. Physical training was recently reported (6) to increase adiponectin receptor expression and plasma adiponectin levels in individuals with impaired glucose tolerance and diabetes.

Because exercise and diet are important prevention and treatment modalities for insulin resistance, this study set out to determine the influence of endurance exercise with or without moderate caloric restriction on insulin resistance, plasma adiponectin, adiponectin multimer distribution, and skeletal muscle adiponectin receptor expression in older obese adults diagnosed with impaired glucose tolerance (IGT). The study design

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enabled the independent effect of exercise as well as the assessment of the combined influence of exercise and diet on these parameters. The aim was to further highlight a role for adiponectin in determining insulin sensitivity. We hypothesized that a lengthy exercise/diet intervention would enhance insulin sensitivity and that this improvement would be accompanied by an increase in serum adiponectin level, multimer distribution, and skeletal muscle adiponectin receptor expression.

**EXPERIMENTAL PROCEDURES**

**Subjects**

A total of 21 older (mean age >60 yr) subjects (males = 7, females = 14) who were weight stable (<2 kg/6 mo weight change), sedentary (<20 min of exercise twice/wk), and obese [body mass index (BMI) 30–40 kg/m²] volunteered to participate in a randomized 12-wk supervised aerobic exercise and diet study (Table 1). All subjects had IGT in accordance with the American Diabetes Association diagnostic criteria. All subjects were free of any contraindications against participation in an exercise/diet program, which included smoking, diabetes, or medications affecting carbohydrate metabolism, surgery within the last year, or evidence of acute or chronic disease (cardiovascular, cerebrovascular, liver, renal, hematological, thyroid, or cancer). All women were postmenopausal for ≥1 yr and had not been on hormone replacement therapy for ≥1 yr before study enrollment. This investigation was carried out in accordance with the principles of the Declaration of Helsinki as well as Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects. The Institutional Review Board at MetroHealth Medical Center approved the research protocol. Written, informed consent was obtained from all subjects in accordance with institutional guidelines for the protection of human subjects.

All subjects underwent a medical history and physical examination, which included a resting electrocardiogram, oral glucose tolerance test, and a complete blood profile (glycosylated hemoglobin, lipid profile, liver, renal, and hematological function test). Cardiovascular function was evaluated using an incremental graded exercise stress test.

**Body Composition**

Standing height without shoes was measured to the nearest 1.0 cm, and body weight was measured in kilograms to the nearest 0.1 kg. BMI was subsequently calculated [wt (kg)/ht (m²)]. Body density was determined from underwater weight, and body composition was assessed by hydrostatic weighing. Significantly different from the preintervention measure. *

**Aerobic Capacity Test (Maximal Oxygen Consumption)**

Maximal oxygen consumption (VO₂ max) was determined during an incremental graded treadmill exercise test. Expired air was analyzed using a Sensor Medics (Palo Alto, CA) 2900Z metabolic system. The test was deemed satisfactory if at least three of the following criteria were attained: plateau in oxygen consumption with increasing workloads, volitional fatigue, a heart rate within 10 beats/min of the age-predicted maximum, or a respiratory exchange ratio >1.10.

**Exercise and Diet Intervention**

All exercise sessions were supervised by an exercise physiologist and conducted in the General Clinical Research Center (GCRC). Subjects were randomly assigned to aerobic exercise (5 days/wk for 60 min/day at 80–85% maximum heart rate) combined with either a hypocaloric diet (ExHypo, involving ~500 kcal reduction, n = 11, 4 men and 7 women) or a eucaloric diet (ExEu, no reduction in caloric intake, n = 10, 3 men and 7 women) for 12 wk.

During the 12-wk intervention, the ExEu group was instructed to follow a weight maintenance diet that consisted of their usual food consumption (~1,800 kcal/day). In contrast, the ExHypo group was instructed to follow a diet with total energy content calculated to reduce body weight by 10–15% (~1,300 kcal/day). Subjects in both groups received a 1-h diet counseling session at weeks 0, 3, 6, 9, and 12 along with evaluation of adherence to the diet as confirmed by 3-day diet records.

**Euglycemic Hyperinsulinemic Clamp**

Prior to all clamp procedures, subjects resided in the GCRC for 3 days and nights and were provided with a weight maintenance diet (55% carbohydrate, 30% fat, and 15% protein). Following an over-night fast (10–12 h), a polyethylene catheter was inserted into an antecubital vein for infusion of insulin (40 mU·m⁻²·min⁻¹), glucose (5.0 mM glucose), and [6,6-³H]glucose, as previously described (21). A second catheter was inserted retrogradely into a dorsal hand vein, which was warmed in a heated box (60°C) for sampling of arterialized venous blood. Blood samples for adiponectin, glucose, insulin, and glucose kinetics were collected and stored at −80°C for subsequent analysis.

**Total Adiponectin and Multimer Analysis**

Serum adiponectin was measured by immunoassay (catalog no. DRP300; R&D Systems). The average intra-assay and interassay coefficients of variation were 7.1 and 7.0%, respectively. The manufacturer’s directions were followed for each assay, and all samples were assessed in duplicate.

The relative amount of adiponectin multimers was determined by Western blot analysis and densitometer quantification. Western blot nondenaturing conditions were followed, which allowed for the detection of at least six immunoreactive adiponectin isoforms, as initially demonstrated by Waki et al. (35). The various multimers have been routinely categorized into approximately >220 KDa, ~120 KDa, and ~70 KDa and designated as high-molecular-weight (HMW), middle-molecular-weight (MMW), and low-molecular-weight (LMW) multimers, respectively.

Table 1. Subject characteristics before and after the exercise/diet interventions

<table>
<thead>
<tr>
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<th>Hypocaloric (n = 11)</th>
<th>Eucaloric (n = 10)</th>
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<tbody>
<tr>
<td></td>
<td>Preintervention</td>
<td>Postintervention</td>
</tr>
<tr>
<td>Age, yr</td>
<td>67.4 ± 1.3</td>
<td>65.0 ± 1.4</td>
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<tr>
<td>Weight, kg</td>
<td>97.4 ± 4.7</td>
<td>89.3 ± 4.1*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>34.0 ± 1.4</td>
<td>31.3 ± 1.3*</td>
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<tr>
<td>Fat mass, kg</td>
<td>38.7 ± 2.8</td>
<td>32.6 ± 2.6*</td>
</tr>
<tr>
<td>Fat-free mass, kg</td>
<td>58.4 ± 3.6</td>
<td>56.7 ± 3.4†</td>
</tr>
<tr>
<td>VO₂ max, ml·kg⁻¹·min⁻¹</td>
<td>20.9 ± 1.0</td>
<td>24.7 ± 0.9*</td>
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</table>

Values are means ± SE (n = 21, 7 males and 14 females). BMI, body mass index; VO₂max, maximal oxygen consumption. Fat mass and fat-free mass were measured by hydrostatic weighing. Significantly different from the preintervention measure. *P < 0.001; †P < 0.03.
medium-molecular-weight (MMW), and low-molecular-weight (LMW) isoforms, respectively.

The protocol involved excluding reducing agent from the sample buffer and preparation of gel samples at room temperature. Protein electrophoresis (45 µg/well) was carried out on 3–8% Tris-acetate gels (Invitrogen; catalog no. EAA0375B0X) and transferred to nitrocellulose overnight at 4°C. Transfer efficiency was monitored using a prestained HMW protein standard (Invitrogen; catalog no. LC5699) and Ponceau S staining following transfer (22). Membranes were blocked [5% nonfat dried milk in 0.1% Tris-buffered saline-Tween 20 (TBS-T)] for 1 h and probed overnight at 4°C with purified mouse anti-Acrp30 monoclonal antibody (BD Transduction Laboratories; catalog no. 611664) raised against human full-length adiponectin (1:500 dilution, in blocking solution), followed by exposure to anti-mouse IgG peroxidase-conjugated secondary antibody (Sigma; catalog no. A 2304; 1:100,000 dilution in 0.1% TBS-T) for 1 h and probed overnight at 4°C with enhanced chemiluminescence. Multimer quantification was determined on a calibrated imaging densitometer (GS-710; Bio-Rad) using Quantity One 4.1.1 software.

Quantitation of the Relative Expression Levels of AdipoR1 and AdipoR2

Total RNA was extracted using the RiboPure kit (Ambion) from 25 mg of vastus lateralis muscle tissue. cDNA was synthesized from 5 µg of total RNA from pre- and postintervention muscle biopsies using oligo(dT)18 primers. Specific primers were synthesized for both adiponectin receptors (AdipoR1 forward: 5'-aaactgcatccacgccctct-3', reverse: 5'-cagatcagcccagcctaaag-3'; AdipoR2 forward: 5'-ggctgcctcctcttaca-3', reverse: 5'-ttgctcagatgctctg-3'). GAPDH was chosen as the normalizing housekeeping reference gene, since it is reported to be appropriate for endurance exercise studies (25) and appeared to be stably expressed in Western blot analysis of our sample data (not shown). GAPDH PCR amplification was performed using a commercially available GAPDH primer pair set (Biomol International; catalog no. Z-108). Quantitative PCR was performed using Brilliant SYBR Green QPCR master mix (Stratagene; catalog no. 600548) on the Mx3000P real-time PCR system platform (Stratagene). Each sample was amplified in duplicate separately for AdipoR1, AdipoR2, and GAPDH using the following PCR conditions: denaturation 95°C, 30 s; annealing 62°C, 1 min; extension 72°C, 1 min for 40 cycles. The relative amount of target mRNA was determined using the comparative threshold (CT) method by normalizing target mRNA CT values to those for GAPDH (ΔCT). Statistical analysis of real-time PCR data was performed using concentrations generated with ΔCT values.

Calculations and Statistical Analysis

The adiponectin SA ratio has previously been reported (28) and calculated from the mean optical densities of the HMW multimer divided by the sum of mean optical densities of the HMW, MMW, and LMW isoforms. Data were analyzed using the Statview II statistical package (Abacus Concepts, Berkeley, CA). Values are presented as means ± SE. The intervention effect on ExHypo and ExEu groups was analyzed using repeated-measures ANOVA. The Wilcoxon signed-rank test was used to assess the difference in adiponectin receptor expression levels pre- and postintervention for both subgroups combined to have sufficient power to detect an effect. The α-level for statistical significance was set at 0.05.

RESULTS

Effect of Exercise and Diet on Anthropometric Variables

All subjects completed the 12-wk exercise and diet intervention program. Both subject groups had similar representations of men and women [ExHypo, exercise/hypocaloric (n = 11, 4 men and 7 women); ExEu, exercise/eucaloric (n = 10, 3 men and 7 women)] and had similar anthropometric variables at the start of the study (Table 1). Body weight was decreased after both programs [8.1 (ExHypo) and 3.4% (ExEu), P < 0.05], and, as expected, the addition of caloric restriction to exercise (ExHypo) caused greater weight loss (P < 0.0001). Whereas the exercise intervention decreased fat mass by 3.3% (P < 0.05), the addition of caloric restriction to exercise (ExHypo) led to a 16% decrease in fat mass (P < 0.001); however, fat-free mass (FFM) was also slightly reduced in the ExHypo group (2.9%, P < 0.03).

Effect of Exercise and Diet on Insulin Sensitivity

Euglycemic hyperinsulinemic clamp-derived glucose disposal rates (GDRs) were increased after both interventions (ExHypo: 2.5 ± 0.3 vs. 4.4 ± 0.5 mg·kg FFM⁻¹·min⁻¹; ExEu: 2.9 ± 0.4 vs. 4.1 ± 0.4 mg·kg FFM⁻¹·min⁻¹, P < 0.0001; Fig. 1). The improvement in insulin sensitivity was similar between groups despite greater weight and fat loss during the ExHypo intervention. There was no difference in the relative change in insulin sensitivity for men vs. women (P = 0.26).

Adiponectin Analysis

Total serum adiponectin measurements by ELISA. Total adiponectin concentrations were between 3.1 and 15.4 µg/ml, with a mean level of 7.6 ± 0.7 µg/ml. Neither group showed a major alteration in total adiponectin levels when pre- and postintervention levels were compared (ExHypo: 7.6 ± 0.9 vs. 6.6 ± 1.0 µg/ml; ExEu: 7.7 ± 1.2 vs 6.8 ± 1.6 µg/ml). As
expected, women had significantly higher total adiponectin levels than men [female (n = 14): 8.3 ± 2.8 μg/ml; male (n = 7): 6.3 ± 3.4 μg/ml, P < 0.05].

Adiponectin immunoblotting. A similar adiponectin gel pattern (Fig. 2A) was produced, as previously reported by Waki et al. (35). There was no significant difference in the absolute levels of HMW, MMW, or LMW. However, the percent change in multimers after the interventions revealed that the MMW was decreased compared with HMW and LMW (Fig. 2B). Both interventions led to a modest, but significant, improvement in the adiponectin SA ratio (HMW/total, P < 0.05; Fig. 3). The relative change in the SA ratio was not different for men vs. women (P = 0.32). Since there was also no between-group difference in the relative change in the GDR, or the SA ratio, we combined the data for the two groups to determine whether there was an association between these two variables (Fig. 4). Using this approach, we found that the change in the adiponectin SA ratio was significantly correlated with the change in insulin sensitivity measured by the GDR (r = 0.52, P < 0.01).

Adiponectin receptor expression. Skeletal muscle AdipoR1 and AdipoR2 mRNA expression increased 1.9- and 3.5-fold, respectively, relative to GAPDH, following the intervention (both subgroupings combined; Fig. 5). This represented a significant increase in expression levels for both adiponectin receptors from the preintervention stage (AdipoR1 P < 0.03, AdipoR2 P < 0.02).

DISCUSSION

Reduced levels of plasma total adiponectin serves as an indicator of insulin-resistant conditions such as diabetes and obesity (4, 32). Furthermore, those genetically predisposed to diabetes have a lower glucose disposal rate during the hyperinsulinemic euglycemic clamp as well as lower plasma adiponectin concentrations (10). In the present study, we found that 12 wk of supervised exercise did not induce significant changes in total serum adiponectin levels despite decreases in body fat and increases in insulin sensitivity. However, the ratio between the oligomeric forms of circulating adiponectin, expressed as the SA ratio, was significantly increased after both interventions. Our data also show that the increase in adiponectin SA ratio following the exercise/diet interventions significantly correlated (P < 0.01) with the increase in insulin sensitivity. Our data are consistent with a recent finding (7) that diet-induced weight loss is associated with changes in adiponectin oligomer composition. However, the present study provides new knowledge on adiponectin biology and shows for the first time that exercise per se can significantly alter adiponectin multimeric distribution and that this change is related to improvements in insulin sensitivity in older obese adults with abnormal metabolic function.

Although previous reports (16, 39) have indicated that reduced circulating adiponectin levels are partially reversible by weight reduction in obese and insulin-resistant subjects, weight loss and exercise training were shown (18) to successfully decrease insulin resistance without affecting total adiponectin levels. Likewise, data in the present study showed significant decreases in body weight and improvements in aerobic fitness without total adiponectin being affected. This might be explained by suggestions that at least a 10% threshold reduction in body weight is required before an increase in circulatory adiponectin levels is observed (8, 18). Alternatively, as adiponectin circulates in the blood as multimers, the level of one or more of these isoforms might actually be of greater relevance rather than total adiponectin as a whole (33).

![Fig. 2. A: representative nonreducing, non-heat-denaturing Western blot of adiponectin multimeric isoforms in human serum. Membrane probed with purified anti-Acrp30 monoclonal antibody (1:5,000; BD Transduction Labs) raised against mouse full-length adiponectin. Multimer molecular weights for high (HMW), medium (MMW), and low molecular weight (LMW) are ~250 kDa, 160 kDa, and 80 kDa, respectively. B: absolute adiponectin multimer expression based on the %change in measurements obtained from serum samples obtained before and after the respective interventions (Pre/Post/Pre%). Data are means ± SE, ExHypo group (n = 11) and ExEu group (n = 10). *Subgroups were not different, but when the subgroups were combined MMW was significantly decreased compared with HMW and LMW, P < 0.03.]
It is noteworthy that this intervention resulted in an almost equal improvement in total insulin sensitivity in both ExHypo and ExEu subgroupings (despite greater weight and fat mass loss in the ExHypo group). It should be stressed that metabolic improvements can therefore occur without substantial weight and fat reduction, further highlighting the benefit of elevated physical activity even without caloric restriction.

It has been shown (12) that HMW adiponectin strongly correlates with glucose tolerance compared with total adiponectin, and HMW has proven (28) to be more successful than other multimers at reducing blood glucose levels. It has been suggested (12) that the HMW isofrom is the active form of adiponectin, although others (27) show that it acts as a precursor pool that upon cleavage produces an active, more short-lived trimer. Comparisons have been made in several studies of total adiponectin vs. HMW (absolute and SA index) in an effort to determine whether adiponectin complexes offer better correlations with indexes of metabolism and disease (13, 28). Data from the present study suggest that the association between the adiponectin SA ratio and insulin sensitivity is not due to an increase in HMW but instead is a result of a decrease in the MMW oligomer. The mechanism behind this effect is not clear from the present data. However, it has been suggested previously that insulin may be an important factor involved in the interconversion of the different adiponectin isoforms (28). We speculate that a reduction in MMW may lead to less competition between the HMW and LMW oligomers for binding to the adiponectin receptor. Adiponectin function is determined by the interconversion of its oligomerization state, which in turn determines its specific biological action in muscle, adipose tissue, and liver. It has been suggested (28) that the alternative tissue-specific expression pattern of the two adiponectin receptors may contribute to this divergence.

We report elevated gene transcription for both adiponectin receptors in individuals with impaired glucose tolerance after a 12-wk intervention. Others (6) also found increased AdipoR1 and AdipoR2 mRNA expression in skeletal muscle with impaired glucose tolerance and type 2 diabetes after 4 wk of increased physical activity and similarly in normal glucose-tolerant individuals even after 3 h of acute training. Whether this gene expression reflects membrane receptor availability remains to be determined since, due to tissue limitations, a full analysis of the protein expression levels could not be performed in this study. However, data from a subset (n = 6) showed no alteration in protein levels (data not shown). Conclusions cannot be drawn from such a sample set and need to be investigated in future studies. It has also been suggested (34, 37) that adiponectin signaling may be more effective in other tissue such as liver and/or adipose tissue. Clearly, downstream translation events need to be investigated to unravel the full molecular response within skeletal muscle cells. However, the increased expression of both AdipoR1 and AdipoR2 as a result of the exercise intervention is of interest and points to a potential augmentation of adiponectin signaling in skeletal muscle.

Our study highlights the independent beneficial influence of physical activity on insulin resistance. It should be noted that impaired glucose-tolerant subjects are expected to have lower-than-normal levels of circulating adiponectin (24). Therefore, subjects with alternative ranges of adiponectin levels may respond differently to exercise/diet intervention. As expected, our study showed that women had significantly higher total adiponectin levels than men. It has previously been reported (35) that the level of the adiponectin HMW multimer also appears to be higher in women compared with men. As each individual was treated in a pair-wise fashion for comparison purposes, a nonstratified mixed-sex sample group was deemed to be valid for the purposes of this study. Our data were limited to the reliance on high-quality protein band resolution following gel electrophoresis. Alternative methodology such as recent commercially available ELISA kits might be considered for adiponectin multimer measurements in future studies (5).

To conclude, endurance exercise improved insulin sensitivity in older obese adults with little additional benefit to the inclusion of caloric restriction. The alteration in adiponectin multimer ratio with exercise indicates a potential source of increased defense capability even in individuals with metabolic abnormalities. Addressing the crucial issue of adiponectin isoform distribution may lead to an enhanced understanding of adiponectin’s role for future therapeutic intervention.

Fig. 4. Correlation data showing the association between the %change in the SA ratio and the %change in insulin sensitivity as measured by the GDR (r = 0.52, P < 0.01). •, ExEu; ○, ExHypo.

Fig. 5. Adiponectin receptors AdipoR1 and AdipoR2 mRNA expression in skeletal muscle biopsy samples pre- and postexercise intervention. The Wilcoxon signed-rank test was used to assess the difference pre- and postintervention for combined subgroupings. Gene expression relative to GAPDH. *AdipoR1, P < 0.03; AdipoR2, P < 0.02.
nary data for this manuscript were previously reported in abstract form in Diabetes Suppl 1; A267, 2005. Thanks also to the YMCA of Greater Cleveland and the research participants for their cooperation and commitment.

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