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

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Running title: Exercise/diet, adiponectin multimer ratio and receptor expression
Abstract
Circulating adiponectin is reduced in disorders associated with insulin resistance. This study was conducted to determine whether an exercise-diet intervention would alter adiponectin multimer distribution and adiponectin receptor expression in skeletal muscle. Impaired glucose tolerant older (>60 years) obese (BMI, 30-40 kg/m²) men (n=7) and women (n=14) were randomly assigned to 12 weeks of supervised aerobic exercise combined with either a hypocaloric (ExHypo, ~500 kcal reduction, N=11) eucaloric diet (ExEu, N=10). Insulin sensitivity was determined by the euglycemic (5.0 mM) hyperinsulinemic (40 mU·m²·min⁻¹) clamp. Adiponectin multimers (high, middle and low molecular weight, HMW, MMW and LMW, respectively) were measured by non-denaturing Western blot analysis. Relative quantification of adiponectin receptor expression through RT-PCR was determined from skeletal muscle biopsy samples. Greater weight loss occurred in ExHypo compared to ExEu subjects (8.0 ± 0.6%, versus 3.2 ± 0.6%, p<0.0001). Insulin sensitivity improved post intervention in both groups, (ExHypo: 2.5 ± 0.3 vs. 4.4 ± 0.5, and ExEu: 2.9 ± 0.4 vs. 4.1 ± 0.4 mg·kg⁻¹FFM·min⁻¹, p<0.0001). Comparison of multimer isoforms revealed a decreased percentage in MMW relative to HMW and LMW (p<0.03). The adiponectin Sₐ ratio (HMW/Total) was increased following both interventions (p<0.05) and correlated with the percent change in insulin sensitivity (p<0.03). Post intervention adiponectin receptor mRNA expression was also significantly increased (AdipoR1 p<0.03; AdipoR2 p<0.02). These data suggest that part of the improvement in insulin sensitivity following exercise and diet may be due to changes in the adiponectin oligomeric distribution and enhanced membrane receptor expression.

Keywords: glucose intolerance; diabetes; obesity; aging; gene expression
Introduction

Over a decade ago, a novel secretory protein was discovered with homology to collagen, a complement factor subunit, and an obscure protein associated with animal hibernation (31). 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 successfully 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 adiponectins’ 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 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 (10, 34). Physical training was recently reported to increase adiponectin receptor expression and plasma adiponectin levels in individuals with impaired glucose tolerance and diabetes (6).

As 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 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 years) subjects (males = 7; females = 14) who were weight stable (<2 kg/6 mo weight change), sedentary (<20 minutes exercise twice per week) and obese (body mass index (BMI), 30-40 kg/m²) volunteered to participate in a randomized 12 week supervised aerobic exercise and diet study (Table 1). All subjects had impaired glucose tolerance (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 at least 1 year and had not been on hormone replacement therapy for at least 1 year 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, OGTT 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. Body Mass Index (BMI) was subsequently calculated (wt (kg)/ht (m)²). Body density was determined from underwater weight and body composition was calculated using the Siri equation as previously described (26).

Aerobic capacity test (VO₂max)

Maximal oxygen consumption (VO₂max) was determined during an incremental graded treadmill exercise test. Expired air was analyzed using a Sensor Medics 2900Z metabolic system (Palo Alto, CA). 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 (RER) > 1.10.

Exercise and diet intervention

All exercise sessions were supervised by an exercise physiologist and conducted in the GCRC. Subjects were randomly assigned to aerobic exercise (5 days/week for 60 minutes/day at 80-85% HR_{max}) 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 weeks.

During the 12-week intervention, the ExEu group was instructed to follow a weight maintenance diet that consisted of their usual food consumption (~1800 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% (~1300 kcal/day). Subjects in both groups received a 1-hour 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 three days and nights and were provided with a weight maintenance diet (55% carbohydrate, 30% fat and 15% protein). Following an overnight fast (10-12 hour) 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, 6-²H] glucose, as previously described (21). A second catheter was inserted retrograde 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 (catalogue number DRP300 R+D systems). The average intra-assay and inter-assay coefficients of variation were 7.1% and 7.0%, respectively. Manufacturer’s directions were followed for each assay and all samples were assayed in duplicate.

The relative amount of adiponectin multimers was determined by Western blot analysis and densitometer quantification. Western blot non-denaturing 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 ~>220KDa, ~120KDa, and ~70KDa and designated high molecular weight (HMW), 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 (45ug/well) was carried out on 3-8% Tris-Acetate gels (Invitrogen catalogue number EA0375BOX) and transferred to nitrocellulose overnight at 4°C. Transfer efficiency was monitored using a pre-stained high molecular weight protein standard (Invitrogen, catalogue number LC5699), and Ponceau S staining, following transfer (22). Membranes were blocked (5% non-fat dried milk in TBS/T 0.1%) for 1 hour and probed overnight at 4°C with purified mouse anti-Acrp30 monoclonal antibody (BD Transduction Labs, catalogue number 611644) raised against human full length adiponectin (1:500 dilution, in blocking solution), followed by exposure to anti-mouse IgG peroxidase conjugated secondary antibody (Sigma, catalogue number A 2304; 1:100,000 dilution in TBS/T 0.1%) and visualized by 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 25mg vastus lateralis muscle tissue. cDNA was synthesized from 5µg of total RNA from pre and post intervention muscle biopsies using oligo (dT)$_{18}$ primers. Specific primers were synthesized for both adiponectin receptors (AdipoR1 forward: 5’-aaacttagccatcccctcct-3’, reverse: 5’-cagatccaggcctagaaag-3’; AdipoR2 forward: 5’-ggcatgtcccctctcttaca-3’, reverse: 5’-tgttgtccaaatgttgcctgt). GAPDH was chosen as the normalizing housekeeping reference gene as it is reported to be appropriate for endurance exercise studies (25) and appeared to be stably expressed in Western blot analysis of our sample set (data not shown). GAPDH PCR amplification was performed using a commercially available GAPDH primer pair set (Biomol international, catalogue number: Z-108). Quantitative PCR was performed using Brilliant Sybr Green QPCR master mix (Stratagene, catalogue number 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 seconds; annealing 62°C, 1 minute; extension 72°C, 1 minute; 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 (delta Ct). Statistical analysis of real-time PCR data was performed using concentrations generated using delta Ct values.

Calculations and statistical analysis

The adiponectin S$_A$ ratio has been previously reported 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 (28). Data was 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 post intervention for both sub-groupings combined, in order to have sufficient power to detect an effect. The alpha level for statistical significance was set at 0.05.
Results

Effect of exercise and diet on anthropometric variables
All subjects completed the twelve-week exercise and diet intervention program. Both subject groups had a similar representation of men and women (ExHypo; exercise/hypocaloric (N=11, 4 men and 7 women) and 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). While 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 FFM was also slightly reduced in the ExHypo group (2.9%, p<0.03).

Effect of exercise and diet on aerobic capacity
Prior to the intervention, maximal aerobic capacity was similar between ExHypo and ExEu groups (20.9 ± 1.0 and 20.6 ± 0.8 ml·kg⁻¹·min⁻¹, respectively), falling below the 10th percentile for subject age and gender, indicating extreme inactivity and lack of fitness. Following 12 weeks of exercise, aerobic capacity was improved in both groups (ExHypo: 15.7 ± 3.1%, and ExEu: 12.5 ± 1.9%, P<0.001) with a similar fitness improvement in each group (Table 1).

Effect of exercise and diet on insulin sensitivity
Euglycemic-hyperinsulinemic clamp derived glucose disposal rates (GDR) were increased after both interventions (ExHypo 2.5 ± 0.3 vs. 4.4 ± 0.5 mg·kg⁻¹·FFM·min⁻¹, and ExEu: 2.9 ± 0.4 vs. 4.1 ± 0.4 mg·kg⁻¹·FFM·min⁻¹, p<0.0001) (Figure 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 versus women (p = 0.26).
Adiponectin analysis

Total serum adiponectin measurements by ELISA

Total adiponectin concentrations were between 3.1-15.4 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 post intervention levels were compared (ExHypo: 7.6 ± 0.9 vs. 6.6 ± 1.0 µg/ml, and 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 (Figure 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 to HMW and LMW (Figure 2b). Both interventions led to a modest, but significant improvement in the adiponectin S_A ratio (HMW/Total, p<0.05) (Figure 3). The relative change in the S_A ratio was not different for men versus women (p = 0.32). Since there was also no between group difference in the relative change in the GDR, or the S_A ratio, we combined the data for the two groups in order to determine if there was an association between these two variables (Figure 4). Using this approach we found that the change in the adiponectin S_A 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 sub-groupings combined, Figure 5). This represented a significant increase in expression levels for both adiponectin receptors from the pre intervention 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 twelve weeks 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 $S_A$ ratio, was significantly increased after both interventions. Our data also show that the increase in adiponectin $S_A$ 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 that diet-induced weight loss is associated with changes in adiponectin oligomer composition (7). 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.

While previous reports have indicated that reduced circulating adiponectin levels are partially reversible by weight reduction in obese and insulin resistant subjects (16, 39), weight loss and exercise training were shown to successfully decrease insulin resistance without affecting total adiponectin levels (18). 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).

It is noteworthy that this intervention resulted in an almost equal improvement in total insulin sensitivity in both ExHypo and ExEu sub-groupings (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 that HMW adiponectin strongly correlates with glucose tolerance compared to total adiponectin (12), and HMW has proved to be more successful than other multimers at reducing blood glucose levels (28). It has been suggested that the HMW isoform is the active form of adiponectin (12) while others show that it acts as a precursor pool, which upon cleavage produces an active more short-lived trimer (27). Comparisons have been made in
several studies of total adiponectin versus HMW (absolute and S_A index) in an effort to determine whether adiponectin complexes offer better correlations with indices of metabolism and disease (13, 28). Data from the present study suggests that the association between the adiponectin S_A 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 that the alternative tissue-specific expression pattern of the two adiponectin receptors may contribute to this divergence (28).

We report elevated gene transcription for both adiponectin receptors in individuals with impaired glucose tolerance after a 12-week intervention. Others also found increased AdipoR1 and AdipoR2 mRNA expression in skeletal muscle with impaired glucose tolerance and type 2 diabetes after 4 weeks of increased physical activity and similarly in normal glucose tolerant individuals even after three hours of acute training (6). Whether this gene expression reflects membrane receptor availability remains to be determined as 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 that adiponectin signaling may be more effective in other tissue such as liver and/or adipose tissue (34, 37). Clearly downstream translation events need to be investigated in order 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 been previously reported that the level of the adiponectin HMW multimer appears to be also higher in women compared to men (35). As each individual was treated in a pair-wise fashion for comparison purposes, a non-stratified mixed-gender sample group was deemed to be valid for the purposes of this study. Our data was 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.
Acknowledgements

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Figure 1. The effect of exercise alone (ExEu) and exercise combined with caloric restriction (ExHypo) on insulin sensitivity as measured during the euglycemic-hyperinsulinemic clamp before and after intervention and represented by the glucose disposal rate (GDR). Data are means ± SE; ExHypo group (n=11), ExEu group (n=10). Units are expressed relative to FFM measured by hydrostatic weighing. *Significant increase from pre-intervention. P < 0.0001.

Figure 2. a) Representative non-reducing, non-heat denaturing Western blot of adiponectin multimeric isoforms in human serum. Membrane probed with purified anti-Acrp 30 monoclonal antibody (1:5000) (BD Transduction Labs), raised against mouse full-length adiponectin. Multimer molecular weights for HMW, MMW and LMW are approximately 250 kDa, 160 kDa and 80 kDa, respectively.

b) Absolute adiponectin multimer expression based on the percentage change in measures obtained from serum samples obtained before and after the respective interventions (Pre-Post/Pre%). Data are means ± SE; ExHypo group (N=11), ExEu group (n=10). *Subgroups were not different, but when the subgroups were combined MMW was significantly decreased compared to HMW and LMW, P<0.03.

Figure 3. The adiponectin S_A ratio, represented by S_A = (HMW/Total)*1000. The bar graph represents the change in adiponectin after each intervention. Both groups had significant improvements in the S_A ratio. *Significant improvement from pre-intervention, p < 0.05

Figure 4. Correlation data showing the association between the percent change in the S_A ratio and the percent change in insulin sensitivity as measured by the glucose disposal rate (r=0.52, p<0.01). ■ ExEu, □ ExHypo

Figure 5. Adiponectin receptors AdipoR1 and AdipoR2 mRNA expression in skeletal muscle biopsy samples pre and post exercise intervention. The Wilcoxon signed rank test
was used to assess the difference pre and post intervention for combined sub-groupings. Gene expression relative to GAPDH. * AdipoR1 p<0.03; AdipoR2 p<0.02
Table 1 Subject characteristics before and after the exercise/diet interventions

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Values are Means ± SE (N=21, 7 Males, 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 pre-intervention measure, * p<0.001, † p<0.03.
Figure 1

Glucose Disposal Rate (mg/kg·FPM·min−1)

ExHypo  ExEu

* *
Figure 4

![Graph showing the relationship between change in Adiponectin S1 ratio and change in glucose disposal rate.](image)

- Change in Adiponectin S1 ratio (%)
- Change in Glucose Disposal Rate (%)

The graph illustrates a positive correlation between the two variables, with data points plotted along a trend line.
Figure 5

[Bar graph showing relative quantification (ratio) adjusted for GAPDH for AdipoR1 and AdipoR2, comparing pre and post conditions. Asterisks indicate significant differences.]