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Effect of obesity and exercise on the expression of the novel myokines, Myonectin and Irisin

Metabolic dysfunction in skeletal muscle is a major contributor to the development of type 2 diabetes. Endurance exercise training has long been established as an effective means to directly restore skeletal muscle glucose and lipid uptake and metabolism. However, in addition to the direct effects of skeletal muscle on glucose and lipids, there is renewed interest in the ability of skeletal muscle to coordinate metabolic activity of other tissues, such as adipose tissue and liver. The purpose of this study was to examine the effects of endurance exercise on the expression level of two novel muscle-derived secreted factors, or myokines, Myonectin and Fibronectin type III domain containing 5 (Fndc5), the precursor for Irisin. **Methods:** We used the diaphragm muscle from both the obese Zucker rat (OZR) and lean Zucker Rat (LZR) with 9 weeks of aerobic training on a motorized treadmill. We examined the gene expression of 12 commonly used reference genes and performed quantitative real-time PCR analysis on the gene expression of Myonectin and Fndc5. **Results:** Of the 12 commonly used PCR reference genes tested we were able to establish that Hypoxanthine phosphoribosyltransferase 1 (HPRT1) and lactate dehydrogenase A (Ldha) remained stable in the diaphragm muscle regardless of obesity or exercise training. Interestingly, we also concluded that the commonly used reference genes: beta-Actin, beta-2-microglobulin, Non-POU domain containing, octamer-binding, Peptidylprolyl isomerase H, 18S ribosomal rna, TATA box binding protein and Transferrin receptor were all found to be altered by the combination of exercise and obesity. Our study showed that the diaphragm muscle of the OZR had significantly higher expression levels of both myonectin and Fndc5. Exercise training had no effect on the expression level of Fndc5, but significantly lowered the gene expression of myonectin in both the LZR and OZR groups. **Conclusion:** Contrary to prior findings regarding the regulation of Fndc5 and myonectin we show that myonectin and Fndc5 expression are both increased in the OZR model of obesity. Further, long-term exercise training decreases myonectin levels, which is opposite, the effect reported with

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short-term exercise. However, this report confirms earlier work showing that Fndc5 gene expression is not altered by chronic exercise.

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Effect of obesity and exercise on the expression of the novel myokines, Myonectin and Irisin

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Running head: Long-term exercise training reduces myonectin levels

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Metabolic dysfunction in skeletal muscle is a major contributor to the development of type 2 diabetes. Endurance exercise training has long been established as an effective means to directly restore skeletal muscle glucose and lipid uptake and metabolism. However, in addition to the direct effects of skeletal muscle on glucose and lipids, there is renewed interest in the ability of skeletal muscle to coordinate metabolic activity of other tissues, such as adipose tissue and liver. The purpose of this study was to examine the effects of endurance exercise on the expression level of two novel muscle-derived secreted factors, or myokines, Myonectin and Fibronectin type III domain containing 5 (Fndc5), the precursor for Irisin.

Methods: We used the diaphragm muscle from both the obese Zucker rat (OZR) and lean Zucker Rat (LZR) with 9 weeks of aerobic training on a motorized treadmill. We examined the gene expression of 12 commonly used reference genes and performed quantitative real-time PCR analysis on the gene expression of Myonectin and Fndc5.

Results: Of the 12 commonly used PCR reference genes tested we were able to establish that Hypoxanthine phosphoribosyltransferase 1 (HPRT1) and lactate dehydrogenase A (Ldha) remained stable in the diaphragm muscle regardless of obesity or exercise training. Interestingly, we also concluded that the commonly used reference genes: beta-Actin, beta-2-microglobulin, Non-POU domain containing, octamer-binding, Peptidylprolyl isomerase H, 18S ribosomal rna, TATA box binding protein and Transferrin receptor were all found to be altered by the combination of exercise and obesity. Our study showed that the diaphragm muscle of the OZR had significantly higher expression levels of both myonectin and Fndc5. Exercise training had no effect on the expression level of Fndc5, but significantly lowered the gene expression of myonectin in both the LZR and OZR groups.

Conclusion: Contrary to prior findings regarding the regulation of Fndc5 and myonectin we show that myonectin and Fndc5 expression are both increased in the OZR model of obesity. Further, long-term exercise training decreases myonectin levels, which is opposite, the effect reported with short-term exercise. However, this report confirms earlier work showing that Fndc5 gene expression is not altered by chronic exercise.

Key Words: Aerobic Training, Fatty Acids, Myonectin, Irisin, Metabolic Syndrome

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Obesity and diabetes are the top health problems in the developed world, and major contributors to the development of cardiovascular disease (1) . Skeletal muscle metabolism is an important regulator in control of whole body glucose and lipid homeostasis. Further, the reduction in insulin-mediated skeletal muscle glucose uptake has long been recognized to be an important underlying mechanism of type 2 diabetes (2) . Lifestyle modification, specifically increased physical activity, has demonstrated enormous therapeutic potential to reverse skeletal muscle insulin resistance.

While the direct role of skeletal muscle metabolism in regulating glucose and lipid metabolism is well established, the potential endocrine-like functions of skeletal muscle to influence glucose and lipid metabolism in other tissues have only recently begun to be investigated. With the advent of proteomics, a number of muscle-derived secreted factors, collectively called myokines, have been identified (3, 4) . Interleukin 6 (IL-6) is the first and most well studied myokine, and it is increased with exercise (5-7) . IL-6 acts to stimulate hepatic glucose production and enhances glucose uptake by insulin-sensitive tissues (8, 9) . These studies have provided the first endocrine-like function of skeletal muscle and established a link between exercise and systemic metabolic parameters (10) . In addition to IL-6, a large number of other muscle-derived secretory proteins have been identified. The purpose of this paper was to examine the impact of obesity and chronic exercise training on two of these novel myokines: 1) fibronectin type III domain containing 5 (FNDC5), the precursor for irisin, and 2) C1q TNF related Protein 15, hereafter referred to as myonectin. FNDC5 is a membrane protein that is cleaved and it's proteolytic cleavage product is secreted as the hormone, irisin (11) . Myonectin is a newly discovered protein with a characteristic C1q domain sequence shared by proteins within the novel CTRP protein family (12) .

Both myonectin and irisin have been linked to improved metabolic health outcomes. Myonectin lowers circulating levels of free fatty acids by increasing uptake in adipose and liver tissues (12) . Whereas, elevated levels of irisin results in increased energy expenditure in mice (11) . To date, circulating levels of both myonectin and irisin have been found to be down-regulated with obesity (12-14) and increased with acute exercise (11, 12, 15) . Further, acute exercise increases gene expression of myonectin, but not FNDC5 (11, 15) . However, the combined effects of exercise and obesity on the regulation of myonectin and irisin have not been examined. This study may give clues to understanding the mechanism behind the endocrine benefits of regular exercise with obesity.

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Methods

Animals. The diaphragm muscles were kindly provided by the lab of Stephen E. Alway, as reported from a previous study (16, 17) . Briefly, equal numbers of 6-wk-old male Obese Zucker rats (OZR) and lean zucker rats (LZR) (Harlan, Indianapolis, IN) were randomly assigned to control (Control, n = 10) or training (Exercised, n = 10) groups. The OZR is a genetic model of obesity due to the presence of the recessive missense mutation (*fa/fa*) in the leptin receptor gene, whereas the LZR has a functioning leptin receptor (Fa/fa or Fa/Fa) (18-21) . Compared to the LZR, the OZR exhibits severe obesity, hyperphagia, hyperinsulinemia, and hyperlipidemia (18-21) . Animals were housed in pathogen-free conditions, two per cage, at 20–22 C with a reversed 12:12-h light–dark cycle, and fed rat chow and water ad libitum throughout the study period. All animal procedures were conducted in accordance with institutional guidelines, and ethical approval was obtained from the Animal Care and Use Committee at West Virginia University.

Training Protocol. LZR and OZR were exercise trained by running on a level motorized rodent treadmill (Columbus Instruments, Columbus, and OH) 5 d/wk for 9 wk, as previously reported (16, 17) . Briefly, during the first 4 wk, the speed of the treadmill and duration of the training sessions was increased gradually from a speed of 10 m/min for 10 min to a final running speed of 20 m/min for the OZR and 24 m/min for the LZR. During the training sessions, mild electrical shock was applied, if necessary, to maintain the animals' running motivation. A slower final running speed was used in the OZR group to compensate for the increased intensity of exercise for these animals that resulted from their greater body weight as compared with LZR. As reported previously, the workload was estimated based on the following formula: Work = 1/2 mass * velocity squared ($W = 1/2m * V^2$). The average body weight of 500 g for the OZR and 350 g for the LZR were used to calculate the treadmill speeds to produce an approximate work output of 0.028 J. These speeds were also reliably maintained by the OZR and LZR, with minimal requirements for external motivation by the investigators. This approach was successful as determined by similar increases in mitochondrial protein content and activity in the trained groups (16, 17) . Animals assigned to the control group were handled daily and exposed to the noise of the running treadmill by placing their cages next to the treadmill during the exercise session.

Tissue collection. Forty-eight hours after the last training session and an overnight fast (~16 h), the animals were anesthetized with injections of pentobarbital sodium (50 mg/kg ip) and euthanized via cardiac puncture. The diaphragm muscles were quickly removed, frozen immediately in liquid nitrogen and stored at -80 C until further analysis.

RNA isolation and reverse transcription. Total RNA was extracted according to standard procedures. Briefly, tissues were homogenized in Trizol reagent (Life Technologies) using a Kinematica polytron in three 30 s bursts, separated by 10 min incubations on ice. After centrifugation at 13.2 rcf, 4 °C for 5 min to remove residual

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particulates, phase separation was accomplished using 3-bromo-5-chloropentane, followed by centrifugation for 15 min at 13.2 rcf, 4 °C. RNA was precipitated from the aqueous phase by mixing with an equal volume 70% ethanol, and then was loaded onto a nucleotide binding column (RNeasy Mini-Kit, Qiagen). On-column DNA digestion was performed using RNase-free DNase (Qiagen) to eliminate residual genomic DNA contamination as per the manufacturer's instructions. RNA was eluted in 50 µl RNase-free water; purity (RIN ≥ 7.0) and concentration were confirmed by microfluidic capillary electrophoresis using an Agilent BioAnalyzer. 1 µg total RNA of each sample was reverse transcribed in a final volume of 20 µl, using GoScript® cDNA synthesis reagents (Promega).

Analysis of reference genes. To screen for potential stable reference genes, an aliquot 1 µl of prepared cDNA from each animal was pooled by group and treatment and the relative content of each reference gene was determined by PCR array according to manufactures directions (RT² Profiler PCR Array; Rat Housekeeping Genes; Qiagen PARN-000ZA). Assuming a perfect efficient reaction, the difference between 1 quantification cycle (Cq) equals a 2-fold difference in starting RNA quantity. Variability of reference genes was deemed to be unacceptable if the maximum difference among the four groups was greater than 0.5 Cq. Reference genes examined are listed in table 1.

Quantitative real time PCR analysis. Validated PCR primers for Myonectin, Fndc5/irisin, Hprt1, Ldha, and RN18S were purchased from SABiosciences (Table 1). A 10-fold dilution series of DNA amplicons generated from an untrained LZR rat muscle was employed as a standard curve for each gene of interest, and the qPCR efficiency was determined for each gene, using a Bio-Rad Cfx thermocycler. Briefly, 0.5 µl of cDNA from the reverse transcription reaction was incubated in appropriate mix (SABbiosciences) for an initial denaturation at 94 °C for 30 s, followed by 40 PCR cycles each consisting of 95 °C for 0 s, 61 °C for 7 s, and 72 °C for 10 s. All qRT-PCR primers displayed a coefficient of correlation greater than 0.99 and efficiencies between 90% and 110%. Data is reported as copy number per amount of starting RNA (0.25 ng per reaction). Specificity of amplification products was further confirmed by analyzing melting curve profiles for primers and products and subjecting the amplification products to agarose gel electrophoresis.

Immunoblot Analysis. Diaphragm muscles were prepared in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) with protease and phosphatase inhibitor cocktails (Sigma). The protein concentration was determined using a Coomassie Plus protein assay reagent (Thermo Scientific). For each sample, 10 µg of protein were loaded and separated on a SDS-polyacrylamide gel, according manufactures direction (BioRad). The proteins were then transferred to Nitrocelluloseous membranes blocked with milk and incubated with Rabbit polyclonal anti-peptide antibody that can recognize myonectin (epitope 77-KQSDKGI NSKRRSKARR-93). Myonectin antibody was kindly provided by the lab of G.W. Wong and had been used previously (12) . Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased

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from Novus Biologicals (NB300-221). Antibody detection was performed with the appropriate horseradish perioxidase-conjugated secondary antibodies. Visualizations were completed with MultiImage III FuorChem ® M (Alpha Innotech) and quantifications were performed by Alphaview Software (Alpha Innotech).

Statistical analysis. Analyses were performed using GraphPad Prism® 5 software package. Student's T-test was used for comparisons between control and exercise trained animals within the same genotype (figure 3). A one-way ANOVA was performed when comparisons were made among all groups (figure 2). Statistical significance was accepted at $P < 0.05$. All data are given as means ± SE. No statistical analysis was performed on the PCR array data of pooled samples (figure 1). A reference gene was deemed acceptable for further analysis if the maximum Cq difference among the pooled samples from the 4 groups was less than 0.5.

Results and discussion

The purpose of this study was to examine the regulation of myonectin and FNDC5 in skeletal muscles of a genetic model of obesity and then to determine the combined effects of obesity and exercise on the regulation of these two proteins. To our knowledge, this is the first study to examine the effect of obesity combined with exercise training on skeletal muscle gene expression of the novel myokines myonectin and FNDC5. Based on the previous literature, we hypothesized that the gene expression of both of these proteins would be reduced with obesity and that the levels would increase with endurance exercise.

Characterization of animals. Our goal in the training regime was to attempt to match final workload between the LZR and OZR. This approach was successful as determined by similar increases in mitochondrial protein content and activity in the trained groups (16, 17) . Although this workload was sufficient to lower body weight (658 ± 18 vs. 502 ± 21, $P < 0.05$) and fasting insulin levels (10.7 ± 1.2 vs. 6.7 ± 1.6 ng/dL, $P < 0.05$) in the OZR, it was not sufficient to induced significant changes in the LZR in these variables, as reported previously (16, 17) .

Identification of appropriate reference genes. Our data was able to confirm that HPRT, HSP90, Ldha, Pgk1, Rplp1, and Sdha remained relatively stable (Cq variability less than 0.5) regardless of obesity or exercise training (Figure 1). However, we also observed that there was greater than 1 Cq difference, among the groups examined, in gene expression of Actb, B2m, and Tfrc (Figure 1 and Table 1). Assuming an efficient reaction, 1 Cq difference represents an approximate 2-fold difference in starting RNA content. This indicates that some commonly used reference genes are effected by the specific set of conditions described in this study and therefore are inappropriate to use as reference genes, normalizing factors that control for equal input of total RNA when performing relative gene expression analyses. Further, these data highlight the importance of exploring the stability of reference genes when performing qPCR analysis.

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Of the stable reference genes, we performed quantitative qPCR analysis of HPRT and Ldha to use as reference genes in our analysis. Quantitative qPCR showed that there was no significant difference between the starting copy number of HPRT and Ldha among the groups in our analysis (Figure 2D and Figure 2E). Interestingly, the reference gene 18S ribosomal RNA (RN18S) also examined by quantitative PCR analysis. RN18S was found to be significantly elevated in the obese exercised group compared to all other groups.

Effect of Obesity on Myonectin and Fndc5. It has been previously documented that circulating levels of both myonectin and irisin are downregulated with obesity and type 2 diabetes (12-14, 22) . Contrary to what we expected from the literature, we found that the OZR had significantly higher expression levels of both myonectin and Fndc5 compared with the LZR (Figure 2A and 2B). There are two possibilities to explain this data: 1) it is possible that both FNDC5 and myonectin are regulated by leptin, as obesity occurs in the OZR model due to a nonfunctioning leptin receptor, any receptor-mediated leptin specific regulation would be absent; 2) a diet high in fat may induce the expression of both FNDC5 and myonectin. Previous work has demonstrated that lipids can increase myonectin expression both *in vivo* and *in vitro* (12) . In the OZR model of obesity, animals are fed a chow diet and become obese due to excessive caloric intake (18-21) . Nevertheless, this finding may be serendipitous as these myokines have yet to be examined in a model with dysfunctional leptin signaling. These observations deserve more extensive analysis than was possible within the scope of the current study.

The combined effects of Obesity and exercise training on Myonectin expression. It has been previously documented that short-term exercise exposure increases gene expression of myonectin (12) . Although myonectin expression had not yet been examined after long-term exercise exposure, we were surprised to find that chronic exercise reduced myonectin expression regardless of obesity status (figure 2A). Because our findings were contrary to what we anticipated, we also examined the muscle protein content of myonectin (figure 3). These results were even more confounding, since, although myonectin gene expression was reduced with exercise, myonectin protein content was elevated with exercise, regardless of obesity. Unfortunately, serum samples were no longer available from these animals to determine the changes to circulating myonectin levels. However, these data suggest that myonectin may act in an autocrine fashion to regulated it's own expression, as has been noted recently for irisin (23) and for other myokines such as IL-6 (24) .

The combined effects of Obesity and exercise training on Fndc5 gene expression. According to the literature neither acute nor chronic exercise alters FNDC5 gene expression (11, 15) . Fndc5 is the precursor for Irisin, and it has been suggested that exercise causes cleavage of Fndc5, releasing irisin and driving the exercise-induced 'browning' of white adipose tissue (11, 25) . This indicates that Fndc5 levels may not be directly regulated by exercise. However, because Fncd5 levels are reduced with obesity and insulin resistance we expected to see a restoration of Fndc5 levels with exercise training in the insulin resistant obese OZR. Although we

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confirmed that exercise did not effect Fndc5 gene expression, contrary to the literature we found that Fndc5 mRNA levels were elevated in the OZR (figure 3B). As stated earlier, this finding indicates that Fndc5 may be regulated by leptin or by dietary fat content.

Conclusion. Both myonectin and irisin have been linked to improved metabolic health outcomes. Myonectin coordinates lipid homeostasis in liver and adipose tissue with the metabolic demands of skeletal muscles (12) , whereas, irisin increases energy expenditure in mice through the browning of white adipose tissue (11) . To date, the combined effects of exercise and obesity on the regulation of myonectin and irisin have not been examined. This study shows that in the OZR both myonectin and Fndc5 gene expression are elevated. Further, contrary to previous findings, myonectin gene expression was negatively regulated by exercise, regardless of obesity. The findings of this study indicate leptin signaling and high fat diet as a potential novel mechanism in the regulation of these proteins, and these possibilities warrant future study. Unfortunately, serum samples were no longer available to analyze the combined effect of exercise and obesity on the circulating levels of these novel myokines. Circulating levels of these myokines should also be examined in a leptin deficient model.

Acknowledgements. We would like to thank the East Tennessee State University Molecular biology Core Facility for support in completing quantitative Real-time PCR experiments. We would like to thank the Lab of Stephen E Alway for providing the diaphragm muscles used in this study.

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295 **Table 1:** Rat Housekeeping Genes; Qiagen PARN-000ZA. Variability of reference
296 genes was deemed to be unacceptable if the maximum difference among the four
297 groups was greater than 0.5 Cq. Reference genes examined are listed in table 1. Cq,
298 quantification cycle.
299
300

Gene Name	Abbreviation	Maximum Cq Difference
Actin, beta	Actb	1.4
Beta-2 microglobulin	B2m	1.0
Hypoxanthine phosphoribosyltransferase 1	Hprt1	.5
Heat shock protein 90 alpha	Hsp90	.49
Lactate dehydrogenase A	Ldha	.19
Non-POU domain containing	Nono	.78
Phosphoglycerate kinase 1	Pgk1	.36
Peptidylprolyl isomerase H	Ppih	.61
RPLP1 ribosomal protein, large, P1	Rplp1	.44
Succinate dehydrogenase complex, subunit A	Sdha	.33
TATA box binding protein	Tbp	.85
Transferrin receptor	Tfrc	1.13

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304 **Table 2**
305

Gene Name	Abbreviation	Accession #	Catalog number
Hypoxanthine phosphoribosyltransferase 1	Hprt1	NM_012583.2	PPR42247F
18S ribosomal RNA	RN18S	NR_046237.1	PPR72042A
Lactate dehydrogenase A	Ldha	NM_017025	PPR56603
Myonectin; C1q TNF Related Protein 15; Family with sequence similarity 132, member B	Myonectin	XM_001060107.2	PPR68386A
Fibronectin type III domain-containing protein 5; also known as irisin	Irisin	XM_001060505.2	PPR46702A

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309 Figure 1
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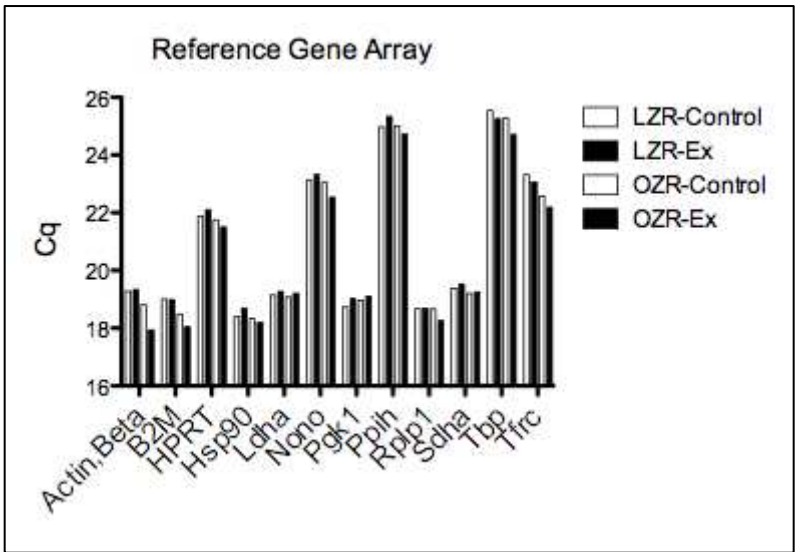

311
312
313 Figure 1. Reference Gene array. To screen reference genes for relative stability
314 pooled cDNA for each group was examined to determine the Cq number by PCR
315 array (RT² Profiler PCR Array; Rat Housekeeping Genes; Qiagen PARN-000ZA).
316 **Abbreviations**: Cq, quantification cycle; LZR, Lean Zucker Rat; OZR, Obese Zucker
317 Rat; ET, Exercise trained; Actb, Actin, beta; B2m, Beta-2 microglobulin; Hprt1,
318 Hypoxanthine phosphoribosyltransferase 1; Hsp90, Heat shock protein 90 alpha
319 (cytosolic), class B member 1; Ldha, Lactate dehydrogenase A; Nono, Non-POU
320 domain containing, octamer-binding; Pgk1, Phosphoglycerate kinase 1; Ppih,
321 Peptidylprolyl isomerase H (cyclophilin H); Rplp1, Ribosomal protein, large, P1;
322 Sdha, Succinate dehydrogenase complex, subunit A, flavoprotein (Fp); Tbp, TATA
323 box binding protein; Tfrc, Transferrin receptor.
324

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Figure 2

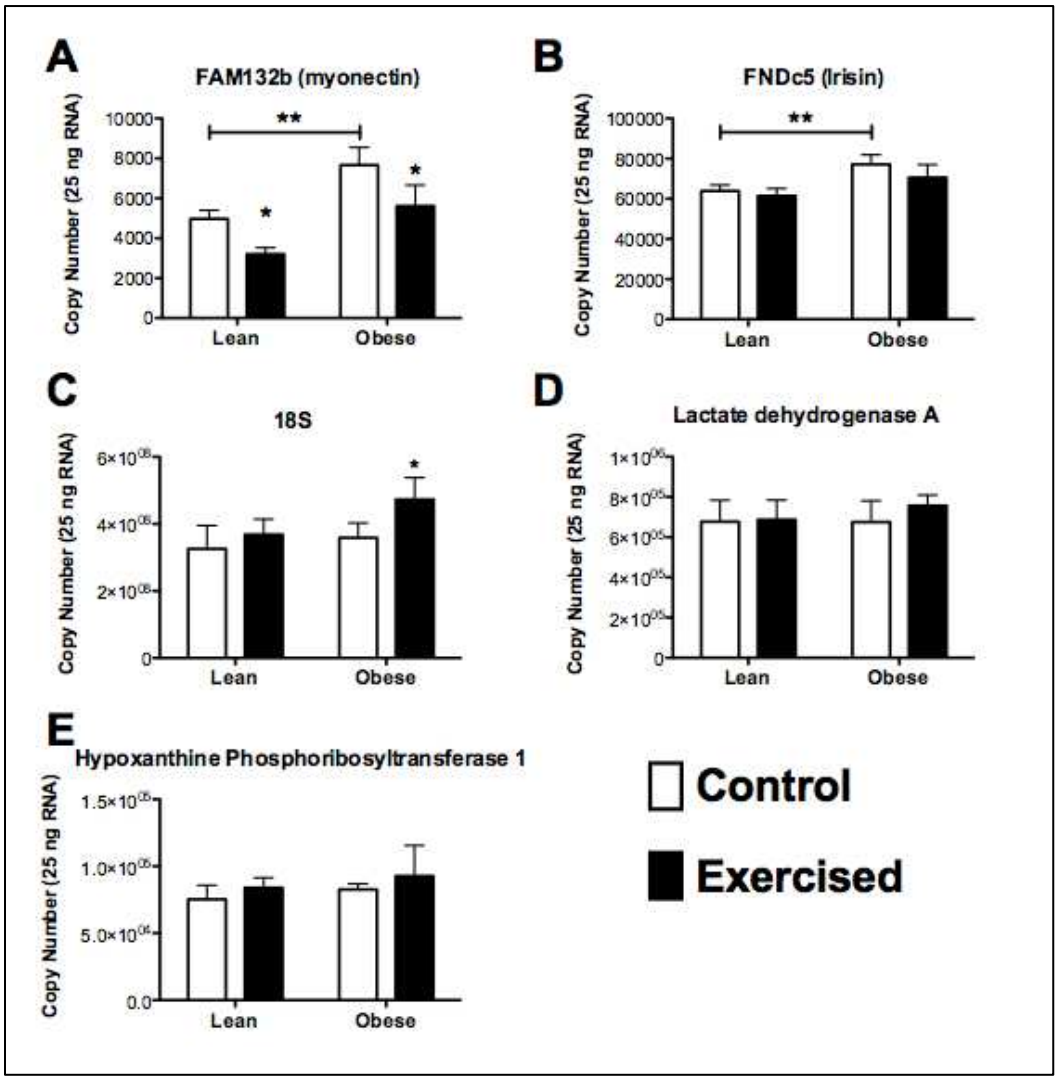

Figure 2: Quantitative RNA analysis. Validated PCR primers for Myonectin, Fndc5/irisin, Hprt1, Ldha, and RN18S were purchase from SABiosciences (Table 1). A standard curve was generated from 10-fold dilution series of DNA amplicons for each gene of interest. All qRT-PCR primers displayed a coefficient of correlation greater than 0.99 and efficiencies between 90% and 110%. Data is reported as copy number per amount of starting RNA. The main effects of obesity (OZR x LZR) and exercise interaction (obesity x exercise) in these animals were analyzed by a two-way ANOVA. Data are presented as means ± SE. *$p<0.05$, data significantly different between control and exercised groups. **$p<0.05$, data from OZR animals was significant different from the LZR animals.

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Figure 3:

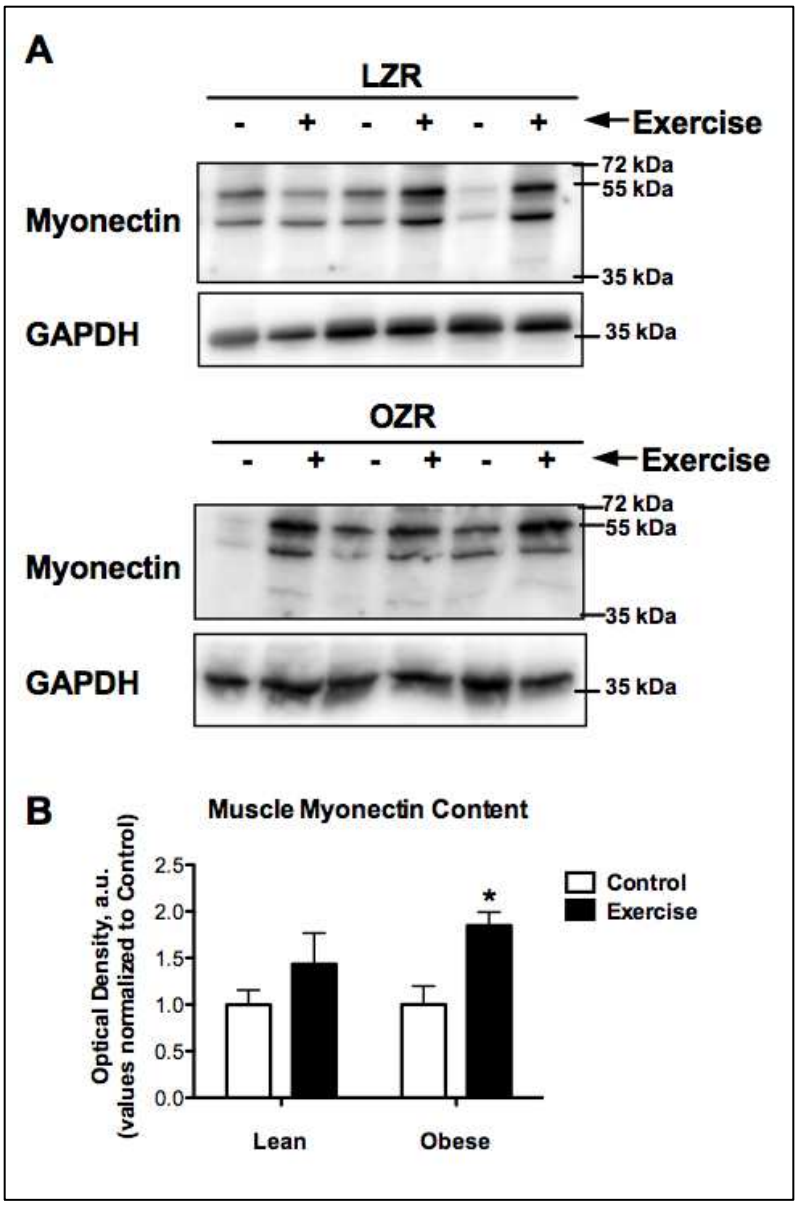

Figure 3: Myonectin/CTRP15 content was examined in the rat diaphragm muscle of the male lean zucker (LZR) and obese zucker rats (OZR). Exercised animals were trained on a motorized treadmill for 12 wk. Control (animals were exposed to the similar environment (positioned next to the treadmill) but were not exercised. The insets show representative Western blots for Myonectin and GAPDH. The data are expressed in arbitrary units with values normalized to mean control value.

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