Gender Dimorphism in Skeletal Muscle Proteome Between Lean and Diet-induced Obese Rats

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Key Words

Gender dimorphism • High fat diet • Muscle fiber type • Muscle proteome • Obesity proteomics

Abstract

Background: Although it has been believed for a long time that gender differences in physiology and metabolism were not relevant beyond the reproductive system, new research has indicated that sexual dimorphism may be more prevalent than previously believed. Therefore, the goal of this study was to develop a global view of the changes in gender-dependent protein abundance in two different types of skeletal muscles (soleus and gastrocnemius) of lean and high fat diet (HFD)-induced obese rats. **Methods:** To examine differential expression of proteins between gender and diet, we performed differential proteome analysis of skeletal muscle from lean and obese rats of both genders fed a HFD using 2-DE combined with MALDI-TOF-MS. **Results:** Our gender-specific proteome comparison showed that male and female rats displayed different patterns of proteome regulation including proteins involved in muscle contraction, carbohydrate and lipid metabolism, oxidative phosphorylation, as well as detoxification and antioxidant defenses. **Conclusions:**

most of the candidate proteins identified herein by differential proteomics were previously unrecognized in gender dimorphism of skeletal muscle. Our data can serve as the basis for specific evidence-based interventions allowing for the prevention and treatment of obesity by matching the different needs of women and men such as the development of gender-based medicine.

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Introduction

Although it has been believed for a long time that gender differences in physiology and metabolism were not relevant beyond the reproductive system, new research has indicated that sexual dimorphism may be more prevalent than previously believed, which has important potential implications in regards to the development of new approaches for the prevention, diagnosis, and treatment of metabolic diseases [1].

In an attempt to understand gender differences in obesity, growing evidence for sexual dimorphism has been revealed, including differences in metabolic rate [2, 3], oxidative capacity [4, 5], energy expenditure [6, 7], and sex hormones [8-10]. However, differences in

susceptibility to obesity between genders still remain largely unknown.

Skeletal muscle represents approximately 40~50% of body weight and is not only involved in contraction, but also is a major site of carbohydrate and lipid metabolism, heat homeostasis, as well as fatty acid oxidation [11]. There are a number of differences between men and women in regards to musculature. For example, males are typically larger and more muscular than females. Skeletal muscle from women shows higher transcriptional activities for fatty acid transport into the skeletal muscle, β -oxidation, and intramyocellular lipid synthesis than men [12].

Muscle fibers are divided into two major types based on their metabolic and contractile nature. Type 1 or slow twitch fibers, which have a slow time to peak tension, are rich in mitochondria and relatively fatigue-resistant. In contrast, type 2 or fast twitch fibers contain two subgroups type 2a (fast twitch, oxidative and glycolytic fibers) and type 2b/x (fast twitch and glycolytic fibers) [11]. Several lines of evidence have shown that fiber type distribution is associated with obesity and insulin resistance [13, 14].

Over the last few years, numerous proteomic investigations have catalogued the skeletal muscle proteins [15-17]. In particular, biomedical investigations into proteome-wide alterations in skeletal muscle tissues have been used to establish biomarker signatures of many muscular disorders, myogenesis, effect of exercise, disuse atrophy, as well as muscle growth and aging, as reviewed by Ohlendieck [18].

Earlier studies demonstrated that sex dimorphism pointed towards a higher tendency of male rats to undergo metabolic syndrome manifestations when exposed to HFD feeding [19]. HFD feeding causes more detrimental effect in males due to higher insulin resistance, lower mitochondrial capacity, and lower facility to adapt to altered metabolic energy conditions [20].

To date, several studies have indicated the existence of gender differences in skeletal muscle, including dimorphism in strength [21], morphometric properties of muscle fibers [22], fatigability [23], and hormonal effects on transcriptome changes [24], mitochondrial activity [20], as well as HFD-induced insulin resistance [19] and oxidative metabolism [25]. To the best of our knowledge, only two proteomics studies concerning gender dimorphism have been reported. Metskas et al. [26] found 26 proteins that were statistically significant different in regards to abundance between genders. They showed that glycolytic enzymes were less abundant in females in

non-exercised murine skeletal muscle. Valle and colleagues [27] analyzed the combined effect of gender and caloric restriction on the protein expression profile in rat liver, and identified 31 sexually dimorphic (15 female- and 16 male-dominant) proteins. However, differentially expressed proteins were interpreted in relation to longevity rather than obesity.

No studies have yet examined gender differences in skeletal muscle proteome in response to HFD, especially with a focus on fiber type dependence. Therefore, the purpose of this study was to develop a global view of changes in protein abundance in the skeletal muscle of lean and HFD-induced obese rats, in order to provide data that can serve as basis for specific evidence-based interventions, which could allow for the prevention and treatment of obesity by matching the different needs of women and men such as the development of gender-based medicine.

Materials and Methods

Animals and breeding conditions

Male and female SLC Sprague-Dawley (SD) rats were purchased from Daehan Experimental Animals (Seoul, Korea) at 5 weeks of age and were housed one per cage in a temperature ($23\pm 2^{\circ}\text{C}$) and humidity (55%)-controlled room with a 12 hr light/dark cycle. Rats were provided free access to standard chow and tap water for an adaption period of 1 week. Male and female rats were randomly divided into two groups, where 20 rats were fed a normal diet (ND, 12% calories from fat; control group) and 20 rats were fed a high fat diet (HFD, 45% calories from fat). Rat feeds were purchased from Feed Korea Lab (Hanam, Korea); the dietary composition of these feeds is shown in Table 1. All rats and feeds were weighed every week for 8 weeks. Rats were food deprived for at least 12 hr before being sacrificed and were anesthetized by 3% diethyl ether. These experiments were approved by the Committee for Laboratory Animal Care and Use of Daegu University. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Blood plasma samples

Blood samples were obtained by resection at the end of the tail of the rat under anesthesia and collected into EDTA-tubes (BD, Franklin Lakes, NJ, USA). Plasma was separated by centrifugation ($3,000 \times g$, 10 min), followed by storage at -80°C until further analysis. Protein concentration in the plasma was determined by the Bradford method [28] using protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA).

Plasma biochemical parameters

Plasma biochemical parameters were measured using plasma of ND-fed rats ($n=7$) and HFD-fed rats ($n=7$) from male

Table 1. Composition of the diets used in this study. ^{a)}Mineral mix; sucrose (118.03 g/kg), calcium phosphate dibasic (500 g/kg), sodium chloride (74 g/kg), potassium citrate (220 g/kg), potassium sulfate (52 g/kg), magnesium oxide (24 g/kg), magnesium oxide (24 g/kg), manganous carbonate (3.5 g/kg), ferric citrate (6 g/kg), zinc carbonate (1.6 g/kg), cupric carbonate (0.3 g/kg), potassium iodate (0.01 g/kg), sodium selenite (0.01 g/kg), chromium potassium sulfate (0.55 g/kg). ^{b)}Vitamine mix, sucrose (981.15 g/kg), thiamine (0.6 g/kg), vitamin E acetate (500 IU/g; 10 g/kg), niacin (3 g/kg), calcium pantothenate (1.6 g/kg), vitamin B12 (0.1%; 1 g/kg), vitamin A palmitate (500,000 IU/g; 0.8 g/kg), pyridoxine (0.7 g/kg), riboflavin (0.6 g/kg), vitamin D3 (400,000 IU/g; 0.25 g/kg), folic acid (0.2 g/kg), menadione sodium bisulfate (0.08 g/kg), biotin (0.02 g/kg). ^{c)}TBHQ, tert-butylhydroquinone.

Ingredient	ND	HFD
Composition by weight, g/kg		
Casein	200	200
Cornstarch	150	155
Sucrose	500	50
Dextrose	0	132
Cellulose	50	50
Soybean oil	0	25
Corn oil	50	0
Lard	0	175
Mineral mix ^{a)}	35	35
Vitamin mix ^{b)}	10	10
TBHQ ^{c)}	0	0.014
DL-Methionin	3	0
L-cystine	0	3
Choline bitartrate	2	2.5
Composition by calories, %		
Protein	21	20
Carbohydrate	68	35
Fat	12	45
Total	3902 kcal/kg	4776 kcal/kg

and female rats, respectively. Commercial kits purchased from Bio Clinical System Corporation (Anyang, Korea) were used for enzymatic measurement of total cholesterol and high density lipoprotein (HDL) cholesterol in plasma, and enzymatic kits obtained from Asan Pharmaceutical (Seoul, Korea) were used for determination of triglyceride (TG) and glucose. An enzymatic fatty acid quantification kit (Abcam, Cambridge, UK) was used to quantify free fatty acids (FFA). A Rat Insulin kit (Millipore Co., Billerica, MA, USA) was used to measure plasma insulin levels, and leptin levels were determined using the Rat Leptin kit (Millipore Co., Billerica, MA, USA), which is a sandwich enzyme-linked immunosorbent assay (ELISA). Sex hormone levels were measured using a Rat Estrogen ELISA kit (Cusabio Biotech. Co., LTD, Wuhan, Hubei, China) and Rat Testosterone ELISA kit (Cusabio Biotech). All measurements were performed according to the manufacturer's instructions.

Quantitative real time RT-PCR (qRT-PCR)

To assess whether we accurately prepared type 1 and type 2 fiber predominant muscle tissues and to compare the expression levels of marker genes specific for type 1 or type 2 muscle fiber, mRNA amounts of the six marker genes were quantified using real-time RT-PCR. The primer sequences, melting temperature and product sizes of the analyzed genes are shown in Table 2. The correct fragment sizes of the PCR products were confirmed using agarose gel electrophoresis (1.5%). Each primer set amplified a single product as indicated by a single peak during melting curve analyses. Both soleus (type 1 predominant) and gastrocnemius (type 2 predominant) muscle mRNA were treated with DnaseI and reverse transcribed by the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed on the Stratagene Mx3000p QPCR system (San Diego, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA). The reactions contained 1× Power SYBR Green PCR Master Mix, 1 µl diluted cDNA template and each primer at 200 nM in a 20 µl reaction volume. After an initial denaturation at 95°C for 10 min, amplification was performed with 40 cycles of 95°C for 15 s, 61°C for 15 s, 72°C for 20 s; plate read; melting curve from 55°C to 95°C, read every 0.2°C, hold for 1 s. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. At the end of the PCR run, melting curves were generated and analyzed to confirm non-specific amplification, the mean value of each triplicate was then used

for further calculations. Gene expression level was normalized to those of Gapdh. For each gene, the sample with the largest ΔC_t value was set as the control. Differences in the expression of target genes between two muscles were analyzed using the *t*-test. The $p < 0.05$ was deemed to be significant and $p < 0.01$ highly significant.

Preparation of skeletal muscle samples for 2-DE

Soleus (type 1 predominant) and gastrocnemius (type 2 predominant) muscle of rats in each group was excised immediately following anesthetization with diethyl ether after overnight fasting. The resulting tissues were then washed with a cold saline solution. Muscle samples were pulverized under liquid nitrogen and stored at -80°C. Tissues were lysed in a 200 µL rehydration buffer solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 1 mM PMSF, 2% IPG buffer (Ampholyte 3-10, Bio-Rad), and a trace of bromophenol blue. A homogenizer (PT 1200E, Kinematica Ltd., Luzern, Switzerland) was used on ice. Extracts were centrifuged at 13,000 ×g for 20 min; the supernatant was then stored at -80°C until analysis. Protein concentration in the muscle tissues was determined by the Bradford method [28].

2-DE analysis

2-DE was performed three times using pooled muscle protein samples from seven rats per group, which consisted of the control group, who were fed an ND, and the obese group, who were fed a HFD. The same diet was provided to both male and female rats. 2-DE experiments were conducted using previously described methods [29]. Briefly, immobilized pH gradient (IPG)-isoelectric focusing (IEF) of muscle samples was performed on pH 3-10 and 18 cm IPG DryStrips (GE Healthcare, Buckinghamshire, UK) in a PROTEIN IEF cell (Bio-Rad, Hercules, CA, USA) using the protocol recommended by the manufacturer. IPG strips were rehydrated passively for 24 hr in strip holders with 350 µL of rehydration solution, which included 7 M urea (Bio Basic, Ontario, Canada), 2 M thiourea (Sigma-Aldrich, St. Louis, MO, USA), 4% CHAPS (Bio Basic), 1 mM PMSF (Sigma-Aldrich), 20 mM DTT (GE Healthcare), and 2%

Table 2. Sequences of primers used for quantitative real-time PCR. ^{a)}For abbreviated gene names, see abbreviation section. ^{b)}NCBI gene database accession number.

Gene name ^{a)}	Accession No. ^{b)}		Primer sequence (5' - 3')	Product size (bp)	Tm (°C)
<i>Gapdh</i>	NM_017008.3	Forward	AGACAGCCGCATCTTCTTGT	207	56.8
		Reverse	CTTGCCGTGGGTAGAGTCAT		
Type 1 fiber-specific genes					
<i>Myh2</i>	NM_001035252.1	Forward	AAAGAGGCTCCAGGTCCAAT	164	56.2
		Reverse	TGCGTGGTTA GCATCTCTTG		
<i>Myh7</i>	NM_017240.1	Forward	CCTCGCAATATCAAGGGAAA	198	56.8
		Reverse	TACAGGTGCATCAGCTCCAG		
<i>Mb</i>	NM_021588.2	Forward	CTTGGGTACCATCCTGAAGA	170	55.9
		Reverse	GAGCATCTGCTCCAAAGTCC		
<i>Myh2</i>	NM_001135157.1	Forward	GAAACACATGCTGAGCTGGA	170	53.3
		Reverse	TCTGCAATCTGTTCCGTGAG		
Type 2 fiber-specific genes					
<i>Myh1</i>	NM_001135158.1	Forward	GAACAGAAGCGCAATGTTGA	168	56.2
		Reverse	TTCCTCGGCTTGTCTCTTGT		
<i>Tnnc2</i>	NM_001037351.1	Forward	GGGGACATCAGCGTTAAAGA	237	53.9
		Reverse	AATGTAACCGTCTGCGTTCC		

IPG buffer (Bio-Rad), and muscle protein of 100 µg. IEF was executed as follows: 15 min at 250 V, 3 hr at 250–10,000 V, 6 hr at 10,000 V, and then held at 500 V until ready to run the second dimension. After focusing, gel strips were equilibrated in a solution containing 6 M urea, 2% SDS (Generay Biotech, Shanghai, China), 1% DTT, 30% glycerol (Bio Basic), and 50 mM Tris-HCl (pH 6.8) for 20 min, followed by further incubation in the same solution, except for replacement of DTT with 2.5% iodoacetamide (Bio-Rad) for an additional 20 min. Gel strips were then placed onto a 20×20 cm 10% polyacrylamide gel for resolution in the second dimension. Fractionation was performed using the Laemmli SDS-discontinuous system, at a constant voltage of 15 mA per gel for 14 hr. For image analysis and peptide mass fingerprinting (PMF), a total of 24 gels, including 3 gels per group with separated protein were visualized by silver staining. Silver staining was performed as follows. Gels were fixed over a period of 30 min in 50% ethanol (Duksan Pure Chemicals, Ansan, Korea) and 5% acetic acid (Duksan Pure Chemicals), followed by 10 min in 30% ethanol. The gels were then water-washed for 5 min three times. Gels were sensitized for 10 min in 0.02% sodium thiosulfate (Sigma-Aldrich), followed by 0.5 min water washes three times, and incubated for 25 min in 0.3% silver nitrate (Kojima Chemicals, Sayama, Japan). After two 0.5 min water washes, proteins were visualized using the developing solution (3% sodium carbonate; Duksan Pure Chemicals), 0.02% sodium thiosulfate, 0.05% formalin (DC Chemicals, Incheon, Korea), and stopped with 6% acetic acid.

Image acquisition and data analysis

Gels were imaged on a UMAX PowerLook 1120 (Maxium Technologies, Akron, OH, USA) and images were compared using modified ImageMaster 2-D software V4.95 (GE Healthcare). Protein spots were automatically detected using the software and the spots were edited manually if necessary. For each experiment, a reference gel was selected from gels of the normal group and spots detected from the other gels were matched with those in the reference gel. Relative optical density and relative volume were also calculated to correct for differences in gel staining. Each spot intensity volume was processed by background subtraction and total spot volume normalization; the resulting spot volume percentage (%Volume) was used for comparison as described previously [30].

Protein identification

For protein identification, protein spots were excised, digested with trypsin (Promega, Madison, WI), mixed with α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid, and subjected to matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis (Ettan MALDI-TOF Pro, GE Healthcare). Spectra were collected from 350 shots per spectrum over an m/z range of 600–3000 and calibrated by two point internal calibration using trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). Peak lists were generated using the Ettan MALDI-TOF Pro Evaluation Module (ver 2.0.16). The threshold used for peak-picking was as follows: 5,000 for minimum resolution of monoisotopic mass and 2.5 for S/N. The search program MASCOT (Mascot Sever 2.3), which was developed by the Matrixscience (<http://www.matrixscience.com>), was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a fixed modification, oxidation (Met) as a variable modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. MASCOT probability-based MOWSE (molecular weight search) score was calculated for PMF. Protein score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event, and greater than 61 was considered significant ($p < 0.05$).

Western blot analysis

Levels of three proteins identified on a 2-DE protein map together with ten proteins of metabolic importance in muscle were confirmed by immunoblot analysis. Tissue lysates were prepared with RIPA buffer (Sigma-Aldrich), homogenized, and centrifuged at 12,000 $\times g$ for 20 min. The extract was diluted in 5X sample buffer (50 mM Tris of pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, and 0.1% bromophenol blue) and heated for 5 min at 95°C before SDS-polyacrylamide gel electrophoresis (PAGE) using 6, 8, 10, or 12% acrylamide gels. After electrophoresis, samples were transferred to a polyvinylidene difluoride (PVDF, Santa Cruz Biotechnology, Santa Cruz, CA, USA) membrane and blocked for 1 h with TBS (tris-buffered saline)-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 containing 5% skim milk). The membrane was rinsed in 3 changes of TBS-T buffer, followed by incubation for 3 h with a 1:1000 dilution of primary monoclonal antibody anti-GP (Santa

Table 3. Plasma biochemical parameters in each group.

^{a)} Levels of biochemical parameters were averaged in each group and expressed as a mean $\pm$ SEM of seven separate experiments. ^{a)} Statistical significance between male and female rats was determined by a t-test, where p value is * p <0.05 and ** p <0.01 (male ND vs. female ND in the <ND, Male> column; male HFD vs. female HFD in the <HFD, Male> column). ^{a)} Significance between ND and HFD rats was represented by † p <0.05 and †† p <0.01 (male ND vs. male HFD in the <ND, Male> column; female ND vs. female HFD in the <ND, Female> column).

Parameter	ND		HFD	
	Male	Female	Male	Female
Glucose (mg/ml)	2.08 $\pm$ 0.18***	1.64 $\pm$ 0.1*	2.39 $\pm$ 0.24	2.21 $\pm$ 0.58
TG (mg/ml)	0.49 $\pm$ 0.09*	0.47 $\pm$ 0.07*	0.71 $\pm$ 0.23	0.67 $\pm$ 0.19
Total cholesterol (mg/ml)	0.36 $\pm$ 0.16*	0.32 $\pm$ 0.2*	0.6 $\pm$ 0.21	0.54 $\pm$ 0.16
HDL cholesterol (mg/ml)	0.52 $\pm$ 0.11**	0.51 $\pm$ 0.09*	0.32 $\pm$ 0.11	0.35 $\pm$ 0.12
Free fatty acid (imol/ml)	1.78 $\pm$ 0.2*	1.79 $\pm$ 0.14	2.05 $\pm$ 0.26	1.97 $\pm$ 0.25
Leptin (ng/ml)	6.62 $\pm$ 2.62***	2.77 $\pm$ 2.15*	21 $\pm$ 4.28**	11.2 $\pm$ 6.51
Insulin (ng/ml)	1.67 $\pm$ 0.48**	0.57 $\pm$ 0.3	2.43 $\pm$ 0.65	1.5 $\pm$ 1.27
Estrogen (ng/ml)	5.56 $\pm$ 0.55***	8.3 $\pm$ 0.78**	6.39 $\pm$ 0.47**	10.83 $\pm$ 1.44
Testosterone (ng/ml)	1.95 $\pm$ 1.85	0.31 $\pm$ 0.07	0.92 $\pm$ 0.56**	0.35 $\pm$ 0.1

Cruz Biotechnology) anti-vimentin (AB Frontier, Seoul, Korea), polyclonal antibody anti-Mb (AB Frontier), anti- β -actin, anti-UCP1, 2, 3, anti-p-AMPK, anti-catalase, anti-Cyt C, anti-SOD2, anti-GLUT4, MM-CK (Santa Cruz Biotechnology), and anti-FAS (Cell Signaling Technology, Beverly, MA, USA) in TBS-T buffer containing 1% skim milk. After 3 washes, the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-goat IgG, anti-mouse IgG, or anti-rabbit IgG secondary antibody (1:1000, AB Frontier) in TBS-T buffer containing 1% skim milk and developed using enhanced chemiluminescence (ECL; GE Healthcare). Immunoblot analysis was performed by scanning with a UMAX PowerLook 1120 and digitalized using image analysis software (KODAK 1D, Eastman Kodak, Rochester, NY, USA).

Statistical analysis

All experimental results were compared by one-way analysis of variance (ANOVA) using the Statistical Package of Social Science (SPSS, version 14.0K) program; data are expressed as the mean $\pm$ SD. Group means were considered significantly different at p <0.05, as determined using protected least-significant difference (LSD) when ANOVA indicated an overall significant treatment effect.

Results

HFD-induced phenotypes in male and female rats

Male and female rats were randomly divided into two groups, where 20 rats were fed a ND and 20 rats were fed a HFD. Initially, the body weight and food intake of ND and HFD rats were nearly identical for both male and female rats. However, after 2 weeks, the body weights of the HFD-fed rats were significantly higher (p <0.05) than the ND-fed rats at all subsequent time points in both male and female rats (Fig. 1A). Total body weight gain per food intake of the HFD-fed male rats was higher than females by an average of approximately 30% (p =0.001) (Fig. 1B). Various biochemical parameters were the measured in each group (Table 3). The concentration of plasma HDL-cholesterol was found to be significantly

Fig. 1. Body weight profile (A) and food efficiency (B) in male and female rats fed ND or HFD. Data are presented as mean $\pm$ SD for seven rats per group and were estimated using the ANOVA test. Statistical significance between male and female rats was determined by a t-test, where the p value was * p <0.05 and ** p <0.01 and significance between ND and HFD rats was represented by † p <0.05 and †† p <0.01.

lower in HFD rats when compared with ND rats (males, p =0.004; females, p =0.018), whereas total cholesterol (males, p =0.032; females, p =0.044), TG (males, p =0.049; females, p =0.028), and FFA (males, p =0.049) levels were remarkably higher in HFD rats relative to ND rats in both males and females. In particular, plasma glucose levels were found to be higher in male rats than female rats (ND, p = 2.2×10^{-4}). Levels of plasma insulin and leptin in seven rats were also determined in ND and HFD rats. In this analysis, significantly higher average insulin and leptin levels were observed in HFD rats when compared with ND rats (insulin in males, p =0.03; leptin in males, p = 1.9×10^{-5} ; leptin in females, p =0.013), and they were remarkably higher in male rats than female rats (insulin

Fig. 2. Comparison of muscle type-specific gene expression between the genders (males vs. females) and diets (ND vs. HFD). White bars are for normal diet and black bars are for HFD. Panel A shows the type 1 fiber-specific genes and panel B shows the type 2-specific genes. Data are presented as mean values $\pm$ SD of relative mRNA expression levels using pooled muscle cDNA from 7 rats per group. Statistical significance between male (M) and female (F) rats was determined by a *t*-test, where *p* values were **p*<0.05 and ***p*<0.01 and significance between ND and HFD rats was represented by †*p*<0.05 and ††*p*<0.01.

in ND rats, *p*=4.1x10⁻⁴; leptin in ND rats, *p*=0.011; leptin in HFD rats, *p*=0.007). We also investigated the plasma concentration of sex hormones, estrogen and testosterone. The concentration of estrogen was significantly higher in HFD rats than ND rats, in both males and females (males, *p*=0.01; females, *p*=0.003). However, testosterone concentration was higher in ND rats than HFD rats in only male rats. These initial findings prompted us to perform further proteomic studies.

Determination of fiber-specific gene expression

To assess whether we accurately prepared type 1 and type 2 fiber predominant muscles and to compare the expression levels of marker genes specific for type 1 or type 2 muscle fiber, mRNA levels of six marker genes were quantified using real-time RT-PCR. As shown in Fig. 2, four type 1-specific genes (e.g. Myl2, Myh2, Myh7, and Mb) were dominantly expressed in soleus muscle. Similarly, two type 2-specific genes were dominantly expressed in gastrocnemius muscle. These results demonstrated that our isolation procedure for each muscle tissue was correctly executed. Expression of several genes was significantly influenced by HFD feeding.

Global analysis of muscle proteome

To examine differential expression of proteins between gender and diet, 2-DE-based proteomic experiments were conducted three times using pooled muscle samples from seven rats per group. Muscle proteins (100 μ g/gel) were separated by 2-DE using a pH 3-10 IEF strip for the first dimension and 10% (w/v) SDS-PAGE gel for the second dimension. Consequently, approximately 390 (soleus) and 400 (gastrocnemius) individual spots were detected, ranging from 15 to 240 kDa between pH 3 and 10 (Fig. 3). Based on high scores (*p*<0.05) and sequence coverage, a total of 48 proteins showing differential expression between the genders or diets were identified with high confidence by MALDI-TOF/MS and database searches (Table 4). To compare

Fig. 3. Representative silver-stained 2-DE gel images of rat muscle proteome in soleus and gastrocnemius muscles. Differentially regulated proteins in each group are marked with circles and proteins of numbers in gel are listed in Table 4.

Spot No. ^{a)}	Protein	Acc. No. ^{b)}	Theoretical MW (kDa)	Theoretical pI	ND ^{c)}		HFD ^{c)}		Score ^{e)}
					Male (% vol) ^{d)}	Female (% vol) ^{d)}	Male (% vol) ^{d)}	Female (% vol) ^{d)}	
	Soleus muscle								
1	78 kDa glucose-regulated protein precursor	gi 25742763	72.4	5.07	0.09±0.01	0.09±0.01	0.07±0.01	0.07±0.01 ^d	127
2	Heat shock protein 90kDa alpha	gi 40556608	83.5	4.97	0.54±0.04 ^c	0.54±0.07	0.40±0.02	0.37±0.03 ^d	268
3	Kelch repeat and BTB (POZ) domain containing 5	gi 157824186	63.5	5.14	0.13±0.01 ^{cc}	0.13±0.01	0.09±0.01	0.10±0.01 ^d	65
4	Alpha-actinin-2	gi 157951643	104.3	5.31	0.07±0.01 ^{cc}	0.08±0.01	0.04±0.01 ^b	0.05±0.00	141
5	Inner membrane protein, mitochondrial, isoform CRA_a	gi 149036390	86.2	5.67	0.19±0.01 ^{cc}	0.19±0.01	0.14±0.01 ^b	0.20±0.02	107
6	Major vault protein	gi 41055865	96.0	5.43	0.06±0.00 ^c	0.07±0.01	0.04±0.01 ^b	0.07±0.01	147
7	Heat shock cognate 71 kDa protein	gi 13242237	71.0	6.16	0.44±0.03 ^c	0.41±0.01	0.35±0.02	0.37±0.02	131
8	Vimentin, isoform CRA_b	gi 149021114	53.7	5.06	0.46±0.05	0.44±0.05	0.47±0.02	0.44±0.03	143
9	Desmin	gi 38197676	53.4	5.21	0.46±0.09 ^{cc}	0.53±0.18	0.14±0.06 ^b	0.32±0.03	287
10	Coronin, actin binding protein 6, isoform CRA_b	gi 149053464	53.2	5.70	0.20±0.01 ^c	0.23±0.02	0.14±0.02 ^b	0.20±0.03	123
11	Ankyrin repeat domain-containing protein 2	gi 157820209	36.8	5.75	0.37±0.03 ^c	0.39±0.04	0.28±0.01 ^{bb}	0.34±0.01	127
12	Troponin T, slow skeletal muscle	gi 66773805	31.1	5.85	0.59±0.06 ^c	0.45±0.08 ^{bb}	0.42±0.07	0.14±0.06 ^{dd}	144
13	Tripartite motif-containing protein 72	gi 117940031	53.7	5.88	0.09±0.01	0.09±0.02	0.07±0.01 ^{bb}	0.04±0.01 ^{dd}	139
14	Myosin light chain 1	gi 127131	20.5	4.99	0.51±0.04	0.42±0.02	0.56±0.03 ^b	0.36±0.04	85
15	Heat shock protein beta-6	gi 20302069	17.5	6.05	0.21±0.03 ^{cc}	0.21±0.01	0.03±0.02	0.07±0.04 ^d	71
16	Smooth muscle and non-muscle myosin alkali light chain 6B	gi 293348444	229	5.52	0.14±0.12 ^{cc}	0.09±0.03	1.09±0.08 ^{bb}	0.03±0.01	192
17	Malate dehydrogenase, cytoplasmic	gi 15100179	36.6	6.16	0.32±0.04	0.33±0.03	0.27±0.02	0.24±0.03 ^d	87
18	Alpha B-crystallin	gi 57580	19.9	6.84	1.25±0.22 ^c	1.19±0.07	0.47±0.04	0.74±0.27	246
19	Creatine kinase M-type (5 spots)	gi 6978661	43.2	6.58	4.18±0.23 ^c	4.17±0.80	3.22±0.53	3.49±0.49	370
20	Dihydrolipoyl dehydrogenase, mitochondrial precursor	gi 40786469	54.5	7.96	0.36±0.02 ^{cc}	0.39±0.06	0.56±0.02	0.58±0.04 ^d	151
21	Phosphoglucosmutase-1	gi 77627971	61.6	6.14	0.32±0.03 ^{cc}	0.36±0.02	0.21±0.02 ^b	0.31±0.03	260
22	Glycogen phosphorylase, muscle form (5 spots)	gi 158138498	97.7	6.65	0.80±0.12 ^c	0.81±0.11	0.49±0.06	0.62±0.23	250
23	Carbonic anhydrase 3	gi 31377484	29.6	6.89	0.59±0.20	0.61±0.19	0.68±0.17	0.55±0.24	242
	Gastrocnemius muscle								
24	Myomesin 1	gi 293349982	177	5.9	0.15±0.02 ^c	0.14±0.02	0.11±0.01	0.09±0.03	164
25	Myosin-binding protein C, fast-type	gi 157823379	129.7	6.16	0.18±0.02	0.20±0.01	0.15±0.03	0.12±0.01 ^{dd}	194
26	2-oxoglutarate dehydrogenase, mitochondrial precursor	gi 62945278	117.4	6.3	0.16±0.02 ^c	0.16±0.03	0.12±0.01	0.11±0.02	119
27	Carnitine O-palmitoyltransferase 2, mitochondrial precursor	gi 126030487	73.9	6.65	0.11±0.05 ^c	0.18±0.04	0.27±0.01	0.24±0.06	151
28	Glycogen phosphorylase, muscle form (5 spots)	gi 158138498	97.7	6.65	2.49±0.19	2.95±0.21 ^a	2.07±0.28	2.10±0.24 ^d	252
29	WD repeat-containing protein 1	gi 62078997	66.8	6.15	0.09±0.01 ^f	0.10±0.02	0.12±0.01	0.14±0.01 ^d	124
30	Creatine kinase M-type (5 spots)	gi 6671762	43.2	6.58	6.54±0.70 ^c	6.55±0.66	4.81±0.26 ^d	5.38±0.23 ^d	82
31	Troponin T class IVd beta-4	gi 207407	27.9	9.53	0.04±0.01	0.03±0.02	0.07±0.01	0.06±0.02 ^d	69
32	Electron transfer flavoprotein subunit alpha, mitochondrial precursor	gi 57527204	35.2	8.62	0.18±0.04 ^{cc}	0.18±0.06	0.35±0.04	0.32±0.07 ^d	83
33	Electron transfer flavoprotein subunit beta	gi 51948412	27.8	7.6	0.04±0.01 ^c	0.04±0.01	0.08±0.01 ^b	0.05±0.01	83
34	Succinyl-CoA ligase subunit beta, mitochondrial	gi 187469277	47.7	6.06	0.47±0.05	0.38±0.04	0.51±0.04	0.48±0.02 ^d	63
35	NADH dehydrogenase Fe-S protein 1, isoform CRA_b	gi 149046009	74.3	5.75	0.33±0.03 ^c	0.35±0.05	0.25±0.02	0.28±0.03	84
36	NADH dehydrogenase Fe-S protein 2, mitochondrial precursor	gi 58865384	52.9	6.52	0.38±0.05	0.36±0.04	0.31±0.02	0.29±0.02	89

Spot No. ^{a)}	Protein	Acc. No. ^{b)}	Theoretical MW (kDa)	Theoretical pI	ND ^{c)}		HFD ^{c)}		Score ^{e)}
					Male (% vol) ^{d)}	Female (% vol) ^{d)}	Male (% vol) ^{d)}	Female (% vol) ^{d)}	
37	NADH dehydrogenase Fe-S protein 3	gi 157817227	30.3	7.07	0.18±0.03	0.17±0.03	0.13±0.02	0.10±0.02 ^d	154
38	NADH dehydrogenase alpha subcomplex subunit 10, mitochondrial precursor	gi 170295834	40.7	7.64	0.21±0.01 ^{cc}	0.18±0.01 ^{aa}	0.16±0.01	0.17±0.00	103
39	ATP synthase beta subunit	gi 1374715	51.1	4.91	0.11±0.02 ^c	0.09±0.02	0.07±0.01	0.07±0.02	103
40	ATP synthase alpha subunit precursor	gi 6729934	55.3	8.28	0.68±0.06 ^c	0.67±0.03	0.50±0.05	0.48±0.05 ^{dd}	157
41	Beta-enolase (4 spots)	gi 71059715	47.3	6.29	5.33±0.45 ^c	5.28±0.28	4.83±0.25	4.62±0.32 ^d	87
42	Glycerol-3-phosphate dehydrogenase, cytoplasmic	gi 57527919	38.1	6.16	0.82±0.04 ^c	0.82±0.07	0.69±0.02	0.69±0.06 ^d	78
43	L-lactate dehydrogenase B chain	gi 6981146	36.8	5.7	0.36±0.04	0.27±0.02 ^a	0.43±0.04 ^b	0.30±0.04	64
44	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial precursor	gi 56090293	39.2	6.2	0.33±0.06 ^c	0.31±0.01	0.45±0.04	0.46±0.04 ^d	88
45	Tropomyosin beta chain	gi 66730475	32.9	4.66	1.26±0.12	0.76±0.07 ^{aa}	1.25±0.06 ^b	0.69±0.19	108
46	Tropomyosin alpha-1 chain isoform i	gi 78000203	28.5	4.71	2.77±0.16	1.88±0.12 ^{aa}	2.77±0.20 ^{bb}	1.82±0.15	161
47	24-kDa mitochondrial NADH dehydrogenase precursor	gi 205628	26.8	6	0.11±0.02 ^c	0.10±0.02	0.08±0.01	0.09±0.01	106
48	Myosin light chain 3	gi 6981240	22.2	5.03	1.07±0.47	0.68±0.26	1.83±0.24 ^b	0.78±0.38	111

Table 4. Differentially expressed and major muscle proteins in male and female rats identified by proteomic analysis. ^{a)}Spot No. indicates numbers in 2DE-image of Fig. 3. ^{b)}Acc. No. is a NCBI database accession number. ^{c)}Notations for experimental group division: ND, control rats fed ND; HFD, rats fed HFD. ^{d)}Statistical significance was determined by a t-test, where p-values are **p*<0.05 and ***p*<0.01; Nor Male vs. Nor Female ^{a)}*p*<0.05 and ^{aa)}*p*<0.01; HF Male vs. HF Female ^{b)}*p*<0.05 and ^{bb)}*p*<0.01; Nor Male vs. HF Male ^{c)}*p*<0.05 and ^{cc)}*p*<0.01; Nor Female vs. HF Female ^{d)}*p*<0.05 and ^{dd)}*p*<0.01. For each protein, the relative intensity was averaged and expressed as a mean ±SD of three separate experiments. ^{e)}MASCOT probability-based molecular-weight search score calculated for peptide mass fingerprinting. Protein score is -10×log(P), where P is the probability that the observed match is a random event; it is based on the NCBI database using the MASCOT searching program as MS/MS data and protein scores >61 are significant (*P*<0.05).

Fig. 4. Scatter plots comparing global type-specific muscle protein expression profiles between males vs. females in ND (A) and HFD (B). White bars are for normal diet and black bars are for HFD. Expression of muscular proteins of male and female rats were plotted against soleus and gastrocnemius muscles, and these plots were created using all spots that appeared in the three gels of each group except for actin (3 spots). The upper and lower diagonal lines show the 1.5-fold regression lines. X- and Y-axis represent volume (%) of each protein spot.

the protein expression profiles globally between males vs. females or soleus vs. gastrocnemius muscle, scatter plots were constructed using all spots (approximately 1180 individual spots) in the three gels of each group (Fig. 4). To provide better images, actin (3 spots) was not included in the plot. In order to identify whether sex dimorphism in global muscle protein expression pointed towards a higher tendency of male rats to undergo a greater weight gain when exposed to HFD feeding, the plots were presented in two different ways. The first plot was designed to compare spot densities between males and females in soleus and gastrocnemius muscle under the normal diet (Fig. 4A), and the other was to compare soleus and

gastrocnemius muscle proteins in male and female rats under the HFD (Fig. 4B). In this analysis, a larger number of proteins showing higher abundance with fold changes

Fig. 5. Graphical presentation of the statistical evaluation of contractile protein abundance of soleus (sol) and gastrocnemius muscles (gas) between the genders (males vs. females) and diets (ND vs. HFD). White bars are for normal diet and black bars are for HFD. Data are presented as mean values $\pm$ SD of volume density (%) of the changed spot in 3 individual gels using pooled muscle tissue from 7 rats per group. These nine proteins have p values < 0.05 when comparing males and females fed ND and/or HFD. Statistical significance between male (M) and female (F) rats was determined by a t -test, where p values were $^*p < 0.05$ and $^{**}p < 0.01$ and significance between ND and HFD rats was represented by $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$. Arabic numerals in the parenthesis of the bar graphs indicate spot numbers from gel images in Fig. 3.

above 1.5 was observed in soleus muscle of females than males for both ND (Fig. 4A) and HFD rats (Fig. 4B). Conversely, the number of proteins showing higher abundance with fold change above 1.5 was greater in gastrocnemius muscle of males than females for both ND (Fig. 4A) and HFD rats (Fig. 4B).

Comparison of abundance in contractile proteins

We identified a total of 9 myofibrillar filamentous proteins and some showed significant gender-dimorphic expression. Two tropomyosin chains and myomesin 1 were gender-dependently regulated only in gastrocnemius muscle, whereas myosin light chains (MLCs), coronin, and troponin T expression was gender dependent in only soleus muscle (Fig. 5). Most of these proteins were obviously more abundant in male rats, except coronin. In contrast, myosin-binding proteins C (MyBP-C) exhibited gender-dependent expression patterns only in gastrocnemius muscle when exposed to HFD (Fig. 5). It is interesting to note that MLC6B was more highly expressed in HFD male rats (Fig. 5).

Comparison of abundance in metabolic proteins

No significant gender-differences were observed in the protein abundance of identified metabolic proteins, including glycerol-3-phosphate dehydrogenase (GPDH),

2-oxoglutarate dehydrogenase (2-OGD), malate dehydrogenase (MDH), and glycogen phosphorylase (GP) (Fig. 6). However, the protein levels of glycogen metabolic proteins, phosphoglucomutase-1 (PGM1) showed significant gender differences in only HFD rats, with a higher abundance in females under both dietary conditions (Fig. 6).

Comparison of abundance in oxidative and energy-producing proteins

As shown in Fig. 7, our proteomic data indicated no significant gender differences in the levels of proteins contributing to energy release or oxidative phosphorylation, which included ATP synthase subunits (ATP syn), NADH dehydrogenase, creatin kinase (MM-CK), and electron transfer flavoprotein (ETF) subunits. Protein levels of the former three were reduced, whereas those of ETF were increased when exposed to HFD in both genders.

Comparison of abundance in cellular stress proteins

In order to identify the existence of gender differences in the expressions of cellular stress proteins, we compared the protein abundances of these proteins between the genders in response to HFD feeding. Our proteomic data pointed to reduced levels of three heat

Fig. 6. Graphical presentation of the statistical evaluation of metabolic protein abundance of soleus (sol) and gastrocnemius muscles (gas) between the genders (males vs. females) and diets (ND vs. HFD). White bars are for normal diet and black bars are for HFD. Data are presented as mean values $\pm$ SD of volume density (%) of the changed spot in 3 individual gels using pooled muscle tissue from 7 rats per group. These 6 proteins have p values <0.05 when comparing males and females fed ND and/or HFD. Statistical significance between male (M) and female (F) rats was determined by a t -test, where p values were $^*p<0.05$ and $^{**}p<0.01$ and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. Arabic numerals in parenthesis of bar graphs indicate spot numbers from gel images in Fig. 3.

shock proteins (HSP90, 71, and beta-6) and GRP78 after HFD feeding in both genders without any significant gender dimorphism (Fig. 8). Interestingly, HSP beta-6 was expressed only soleus muscle.

Western blot analysis of muscle proteins

To verify these proteomic results, we carefully comparing this data with Western blot analysis of the three proteins of interest. In addition, we also compared protein abundance of some muscular proteins of importance between the genders and dietary conditions, which were not detected in our proteomic investigations. The abundances of GP, MM-CK, and vimentin detected by 2-DE analysis were in accordance with those of Western blot analysis (Fig. 9). The protein levels of FAS were

Fig. 7. Graphical presentation of the statistical evaluation of oxidative and energy-producing protein abundance of soleus (sol) and gastrocnemius muscles (gas) between the genders (males vs. females) and diets (ND vs. HFD). White bars are for normal diet and black bars are for HFD. Data are presented as mean values $\pm$ SD of volume density (%) of the changed spot in 3 individual gels using pooled muscle tissue from 7 rats per group. These 6 proteins have p values <0.05 when comparing males and females fed ND and/or HFD. Statistical significance between male (M) and female (F) rats was determined by a t -test, where p values were $^*p<0.05$ and $^{**}p<0.01$ and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. Arabic numerals in parenthesis of bar graphs indicate spot numbers from gel images in Fig. 3.

higher in males, whereas those of GLUT4, Cyt C, SOD, catalase, UCP1, UCP2, UCP3, and myoglobin were higher in females albeit this was partly dependent upon muscle type (Fig. 10). Expectedly, UCP3 showed markedly greater abundance than those of UCP1 and UCP2, where all three UCPs were dominantly expressed in soleus muscle, with higher levels in females.

Discussion

There is now a consensus that rodent females have a superior muscular capacity compared to males due to higher muscle mitochondrial and protein contents as well as greater oxidative phosphorylative capacities [20, 25].

Fig. 8. Graphical presentation of the statistical evaluation of cellular stress protein abundance of soleus (sol) and gastrocnemius muscles (gas) between the genders (males vs. females) and diets (ND vs. HFD). White bars are for normal diet and black bars are for HFD. Data are presented as mean values $\pm$ SD of volume density (%) of the changed spot in 3 individual gels using pooled muscle tissue from 7 rats per group. These 4 proteins have p values <0.05 when comparing males and females fed ND and/or HFD. Statistical significance between male (M) and female (F) rats was determined by a t -test, where p values were $*p<0.05$ and $**p<0.01$ and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\ddagger}p<0.01$. Arabic numerals in parenthesis of bar graphs indicate spot numbers from gel images in Fig. 3.

However, genomic or proteomic approaches to globally identify gender differences has still not been established to support this consensus.

In this proteomic study, we for the first time identified numerous proteins showing gender dimorphism in skeletal muscle on rats under both control conditions and in response to HFD feeding. Several previous biochemical studies had investigated whether gender dimorphism occurred in mitochondrial function and if oxidative stress leads to differences in the development of HFD-induced insulin resistance in rat skeletal muscle [19, 25].

Most interesting is that our gender-specific proteome comparison showed that male and female rats displayed different patterns of proteome regulation including proteins involved in muscle contraction, carbohydrate and lipid metabolism, as well as oxidative phosphorylation and cellular stress.

Proteins involved in muscle contraction

Myofibrillar filaments are responsible for generating the physical movement of muscles, and our differential

Fig. 9. Validation of differentially regulated muscle proteins of soleus (sol) and gastrocnemius muscles (gas) in male (M) and female (F) rats in response to HFD by immunoblot analysis. White bars are for normal diet and black bars are for HFD. Levels of three proteins identified from 2-DE analysis were established using pooled muscle samples from 7 rats per group. Data are representative of three independent experiments. The Y-axes of the bar graphs for the Western blot analysis refer to protein density normalized by β -actin. Statistical significance between male (M) and female (F) rats was determined by a t -test, where p values were $*p<0.05$ and $**p<0.01$ and significance between ND and HFD rats was represented by $^{\dagger}p<0.05$ and $^{\ddagger}p<0.01$. Arabic numerals in parenthesis of bar graphs indicate spot numbers from gel images in Fig. 3.

proteomic analysis revealed that high fat loading is associated with perturbations of the myofibrillar network. Most noteworthy was the up-regulation of myosin and tropomyosin chains in males, which are the primary

Fig. 10. Differentially regulated muscle proteins of metabolic importance in male (M) and female (F) rats in response to HFD by immunoblot analysis. Sol: soleus muscle, Gas: gastrocnemius muscle. White bars are for normal diet and black bars are for HFD. Levels of 10 important muscle proteins were established using pooled muscle samples from 7 rats per group. Data are representative of three independent experiments. The Y-axes of the bar graphs refer to protein density normalized by β -actin. Statistical significance between male (M) and female (F) rats was determined by a t -test, where p values were * p <0.05 and ** p <0.01 and significance between ND and HFD rats was represented by $\dagger$ p <0.05 and $\ddagger$ p <0.01

determinants of force and velocity in muscle fibers [31]. It is interesting to note that myosin light chain 1 (MLC1) was dominantly expressed in gastrocnemius muscle and showed weak gender differences, whereas MLC3 was dominantly expressed in soleus muscle with significant gender dimorphism. Surprisingly, MLC6b was markedly increased only in HFD male rats, suggesting a possible role in adaption of myofibrils associated with a drastic remodeling of contractile apparatus, or a signature of adaptive fiber shifting towards a more aerobic oxidative metabolism in response to high fat loading (Fig. 5). Therefore, MLC6b can be a potentially useful indicator of a fast-to-slow transformation process in high fat loaded muscles.

Tropomyosin chain α and β also showed sex dimorphism in gastrocnemius but not in soleus muscle, where males showed higher protein levels. However, the levels of these proteins were almost identical before and after HFD feeding. The higher expression levels of these proteins in male rat muscle also support greater contractile force of male rats, but maintenance of their protein levels

after HFD feeding was unexpected and requires further study.

One of the most exciting findings of this proteomic study was the gender-different regulation of several myofibrillar regulatory proteins. Down-regulation of myosin-binding protein C (MyBP-C) in HFD-fed rats in both genders suggests that there was a loss of affinity for myosin resulting in impaired filament assembly and regulation of contraction due to myocyte disarray [32, 33].

Another important observation was the down-regulation of troponin T fast in HFD-fed rats, which was more down-regulated in females. Troponin T specifically controls the position of tropomyosin near the interface between actin and myosin and thus mediates the activation and force development of actomyosin contractile units [31]. Therefore, down-regulated troponin T fast may result in loss of muscle force and contractile speed when exposed to HFD. This pattern was confirmed by the significantly decreased Troponin T fast at both the protein level [26] and transcript level [24] in the muscle of other

obese murine models. The lower proportion of oxidative fibers was consistent with the decreased expression levels of both protein and mRNA of Mb in the HFD rats (Fig. 2 and Fig. 10).

Similarly, the level of myomesin-1 was decreased in HFD-fed rats of both genders. However, coronin (actin binding protein 6) showed higher protein levels in females than males when exposed to HFD; however, we currently have no explanation for these differences (Fig. 5). Interestingly, among the nine contractile proteins and their elements identified here, five were more abundant in soleus muscle and three were dominantly expressed in gastrocnemius muscle. Higher abundances of these proteins do not directly indicate greater muscle strength because an increase in the MLC element has also been found in aged muscle [34]. Our proteomic data of contractile proteins were comparable with the results reported by Gelfi et al. [15], who demonstrated that four rat contractile proteins were more abundant in soleus muscle, whereas five were more dominantly expressed in gastrocnemius muscle under normal dietary conditions. Altogether, down regulation of these myofibrillar regulatory proteins may favor decreased contractile capacities when exposed to HFD in both genders [35, 36]. Our data clearly demonstrated the existence of gender differences in several proteins involved in muscle contraction, which supports the fact that higher forces of motor units in male muscles can be generated due to higher expressions of contractile proteins.

Metabolic proteins

When the carbohydrate load was increased, men produced higher concentrations of muscle glycogen, while women did not. However, women oxidized significantly more lipid and less carbohydrate and protein compared with men during exercise. This impaired ability to compensate for muscle glycogen in response to carbohydrate-loading may result in a higher weight gain in men [21]. Our proteomic data also support this finding; there was a lower protein abundance of PGM1 in HFD-fed male rat muscle, implying a greater myofiber glycogen content (storage capacity) (Fig. 6).

Metskas et al. [26] analyzed the skeletal muscle proteome and found that glycolytic enzymes were less abundant in females, which suggests that females rely on lipid oxidation more than males. However, two glycolytic proteins (GP and β -enolase) identified in this study did not show gender differences and differences were only observed in response to the HFD. We also detected a decreased abundance of two different enzymes involved

in the TCA cycle (2-OGD and MDH) in HFD-fed rat muscle but no significant gender differences were observed. A decreased protein level of MDH reflects reduced lipogenesis in HFD rat muscle due to depressed capacity of providing extra-mitochondrial reducing equivalents from the oxidation of malate [37].

Oxidative proteins

We also attempted to examine gender-different changes in energy production and oxidative phosphorylation. We detected significantly reduced protein levels of both ATP synthase subunits α and β (ATP-syn α and β) in HFD male and female rats without a gender dimorphism. These data suggest a perturbation in ATP synthesis in HFD-fed obese rats, which is consistent with the earlier finding that ATP-syn β played a role in the regulation of ATP synthesis [38].

In accordance with the reduced ATP-syn levels in HFD rats, the protein levels of MM-CK in both fibers were also significantly reduced in both genders when exposed to HFD. Since decreased levels of MM-CK in HFD rats could be explained by reduced amounts of soleus muscle, our data suggest that MM-CK might play a role in mitochondrial fuel oxidation [38]. The reduced oxidative capacity in HFD-fed obese rats in both genders was also supported by the reduced levels of NADH dehydrogenases (Fig. 7)

In contrast, muscular protein abundance of electron transfer flavoprotein (ETF) was increased in HFD-fed obese rats in both genders when compared with ND rats. β -oxidation of fatty acid in mitochondria requires several dehydrogenases and ETF [39]. ETF serves as an obligatory electron acceptor for fatty acid oxidation, and is then transferred to the respiratory chain [40]. It has been shown that expression of ETF and several dehydrogenases involved in β -oxidation are regulated by various factors, including development, hormones, and nutrition [41]. Therefore, the expression level of ETF is an important indicator of the capacity of β -oxidation. Both ETF α and β were more abundant in soleus muscle due to the mitochondrial volume density when compared to gastrocnemius muscle. Moreover, their levels were increased in both genders when exposed to HFD (Fig. 7). This result suggests a shift towards a more aerobic oxidative metabolism in high fat-loaded muscle fibers.

Cellular stress proteins

The reduced abundance of heat shock proteins (HSP90, 71, and B6) in HFD-induced obese rats was unexpected since they function at the cellular level to

protect cells against many chronically and acutely stressful conditions. The HSPs are expressed by cells in response to stresses such as high temperature, free radicals, shear stress, and toxins [42]. They are involved in the renaturation of damaged proteins, allowing them to refold into their native conformation. Indeed, Chung et al. [43] demonstrated that HSP72 blocks inflammation and prevents insulin resistance in the context of genetic obesity or high-fat feeding. However, all three HSPs detected in this study did not show significant gender differences. Similarly, reduced protein levels of 78 kDa glucose-regulated protein (GRP78), which is a HSP, were observed in HFD-fed rat muscle in both genders without any significant differences. It is currently not known which cellular stress induces HSPs and GRP78 in muscle of patients with metabolic syndromes [38]. We believe that the decreased levels of identified HSPs and GRP75 in HFD rats were not the direct cause of cellular stress leading to increased amounts of incorrectly folded and denatured proteins, mediated by high fat loading.

Accumulating evidence suggests that HFD intake increases muscle fatty acid uptake and oxidation, leading to excessive production of reactive oxygen species (ROS) as well as impaired antioxidant defenses. ROS-induced mitochondrial dysfunction in muscle can lead to disruptions of lipid metabolism by increasing intramuscular lipid content and causing insulin resistance. This tendency was likely more severe in males than females, in that two antioxidant enzymes (catalase and SOD2) were more abundant in females in both muscles irrespective of dietary condition (Fig. 10).

A variety of studies have raised the possibility that HFD induced thermogenesis occurs in oxidative muscle [44, 45], and demonstrated that expression of UCPs in skeletal muscle enhanced respiratory uncoupling and glucose transport, thereby preventing HFD-induced obesity [44-47]. Based on these observations, we compared the expression patterns of UCPs in normal and HFD rats between the genders. Surprisingly, in our immunoblot analysis, a higher abundance of UCP1 was observed only in HFD-fed female rat muscle. Ectopic UCP1 expression in skeletal muscle has been achieved mainly in UCP1 transgenic murines [48, 49]. However, Nibbelink et al. [50] found that UCP1 was not specific to BAT and could be expressed in smooth muscle. A subsequent study by Almind et al. [51] revealed that muscular UCP1 expression resulted from ectopic deposits of brown fat cells in intermuscular depots (interspersed). Together with these results, our finding suggest that the high abundance of UCP1 in HFD female rats might play

a role in the reduced body weight gain and decreased insulin resistance through greater muscular thermogenic activity. This UCP1-mediated uncoupling of oxidative phosphorylation in skeletal muscle of HFD female rats also occurred in conjunction with AMPK activation, which can stimulate insulin-mediated glucose uptake in skeletal muscle (Fig. 10).

Meanwhile, both UCP2 and UCP3 were considerably induced in soleus muscle with higher levels in females, particularly in HFD rats. The high abundance of UCP2 and UCP3 in soleus muscle is line with the levels of antioxidant enzymes (catalase and SOD2), suggesting that both ROS production and adaptive thermogenesis were induced by a HFD (Fig. 10). This is also supported by the fact that notable antioxidant enzymes such as SOD2 and catalase were up-regulated. Taken together, female rats under HFD exhibited more thermogenic activity than males via higher expression of UCP1, and displayed higher antioxidative activity through enhanced expressions of UCP2 and UCP3.

Our Western blot analysis also demonstrated that HFD female rats showed higher abundance of mitochondrial cytochrome C oxidase (Cyt C), glucose transporter 4 (GLUT4), as well as antioxidant enzymes (catalase and SOD2) in both soleus and gastrocnemius muscle irrespective of dietary condition. These results support the fact that females have greater mitochondrial oxidative metabolism and a better capacity to counteract metabolic syndromes mediated by oxidative stress. Interestingly, the abundance of fatty acids synthase (FAS) was almost equal between the two muscles, and less abundant in females.

Conclusions

Most of the candidate proteins identified in this study by differential proteomics were previously unrecognized in regards to the gender dimorphism of skeletal muscle. The gender dimorphism found in this proteomic study could point towards a higher tendency of male rats to undergo metabolic syndrome manifestation associated with higher reliance on lipid as an energy fuel, lower antioxidative capacity, decreased energy expenditure despite of higher contractile protein expression. Although we did not clarify whether sex-dimorphic regulation of muscular proteins mainly resulted from hormonal effects, this can be established by using orchietomized and/or arietomized rodent models or sex hormone-treated muscle cell lines in future studies. Our data can serve as

a basis for the development of specific evidence-based interventions allowing for the prevention and treatment of obesity by matching the different needs of women and men such as the development of gender-based medicine.

Abbreviations

AMPK (AMP-activated protein kinase); ATP-syn (ATP synthase subunit); Cyt C (cytochrome C oxidase); 2-DE (two-dimensional electrophoresis); ETF (electron transfer flavoprotein); FAS (fatty acid synthase); Gas (gastrocnemius muscle); GLUT (glucose transporter); GP (glycogen phosphorylase); GPDH (glycerol-3-phosphate dehydrogenase); GRP (glucose-regulated protein); HFD (high fat diet); HSP (heat shock protein); Mb (myoglobin); MDH (malate dehydrogenase); MLC (myosin light chain);

MM-CK (muscle type creatine kinase); MyBP-C (myosin-binding protein C); Myh (myosin heavy polypeptide); Myl (myosin light polypeptide); ND (normal diet); 2-OGD (2-oxoglutarate dehydrogenase); PGM1 (phosphoglucomutase-1); SOD (superoxide dismutase); Sol (soleus muscle); Tnnc (troponin C type); UCP (uncoupling protein)

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