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Effects of Acute Exercise Over Heart Proteome From Monogenic Obese (ob/ob) Mice

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Exercise is recognized to prevent and attenuate several metabolic and cardiovascular disorders. Obesity is commonly related to cardiovascular diseases, frequently resulting in heart failure and death. To elucidate the effects of acute exercise in heart tissue from obese animals, 12-week-old C57BL6/J obese (ob/ob) and non-obese (ob/OB) mice were submitted to a single bout of swimming and had their hearts analyzed by proteomic techniques. Mice were divided into three groups: control (ob/ob, n = 3; ob/OB, n = 3); a moderate intensity consisting of 20 min of swimming around 90% of Maximal Lactate Steady State (ob/ob, n = 3; ob/OB, n = 3), and a high intensity exercise performed as an incremental overload test (ob/ob, n = 3; ob/OB, n = 3). Obesity modulations were analyzed by comparing ob/ob and ob/OB control groups. Differential 2-DE analysis revealed that single session of exercise was able to up-regulate: myoglobin (ob/ob), aspartate aminotransferase (ob/OB) and zinc finger protein (ob/OB) and down-regulate: nucleoside diphosphate kinase B (ob/OB), mitochondrial aconitase (ob/ob and ob/OB) and fatty acid binding protein (ob/ob). Zinc finger protein and α-actin were up-regulated by the effect of obesity on heart proteome. These data demonstrate the immediate response of metabolic and stress-related proteins after exercise so as contractile protein by obesity modulation on heart proteome.

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The last century was characterized by intense changes in lifestyle as a direct response to urban modernization and technology development. Hyper-caloric food intake in conjunction with a sedentary lifestyle now characterize the life of millions of people contributing to overweight and obesity development (WHO, 2011). Obesity is traditionally defined as a multifactor disease, closely related to genetic, neurological, endocrinological and compartmental factors that in sum lead to a positive caloric balance and a further excessive fat accumulation (Kaiyala and Schwartz, 2011). According to the World Health Organization, approximately 2.8 million of adults die every year by pathologies linked to overweight and obesity (WHO, 2011). Unfortunately, the outlook is not positive due the number of obese people is forecast to reach approximately 700 million by 2015 (WHO, 2011). One of the major problems concerning obesity is its proven link with other pathologic conditions such as diabetes, hypertension and, especially, cardiovascular diseases such as heart failure (Kenchaiah et al., 2002). Together, cardiovascular disease and obesity are responsible for one of the main causes of death in the world (WHO, 2011).

Nevertheless, the mechanisms by which obesity impairs heart and cardiovascular function are not fully understood. However, it has been pointed out that these mechanisms affect the heart's metabolism and contractile structures, leading to a less functional heart (Abel et al., 2008). High-rates of serum free fatty acid (FFA) and triglycerides may induce a certain heart inability to use other energy substrates, been this process closely related to mitochondrial oxidative impairment (An and Rodrigues, 2006). Along this process, ectopic fat accumulation around the heart leads also to lipid infiltration into the myocardium, inducing the steatosis process, a key mechanism for tissue inflammation. By this way heart suffers with lipotoxicity, a typical phenotype resultant of chronic obesity (Wende and Abel, 2010). Along lipotoxicity, heart suffers high hemodynamic resistance, a maladaptive process that, when chronically imposed, may lead to myocardial pathologic hypertrophy, characterized by collagen infiltration, up-regulation of myofibrilar proteins and shifting on the proportion of α and β-myosin isoforms (Diez et al., 2005).

Molecular research is leading to a more accurate and better understanding of the mechanisms by which obesity modulates the heart's phenotype. This knowledge is essential to target new clinical molecular biomarkers and also for pharmacological development. Once the pathogenesis of heart obesity is known, the scientific community can better understand how the obese heart responds to non-pharmacological and alternative treatment, such as exercise. It is widely known that exercise has a restorative and beneficial effect on cardiovascular

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improvement been recommended for several other diseases treatment and health maintenance. It has been demonstrated that exercise training could have a direct effect over the regulation of myofibrilar and mitochondrial proteins, leading the heart to a higher oxidative and resistant capacity (Bansal et al., 2010). This molecular modulation seems to have a direct link to exercise intensity, which is a determinant factor from the distinct process of heart adaptation and maladaptation. Thus, several research has focused on the heart adaptation by chronic stimulus of training (Petriz et al., 2011), been only few proteomic investigations focusing on the acute physiologic responses to exercise (Gonzalez and Manso, 2004). Moreover, none of these studies has focused on heart tissue.

Therefore, research on the heart's acute responses to exercise may lead to further information about how this organ deals with chronic stimulus and its response to different exercise intensities. These responses may also lead to a better approach in treating different cardiac disorders, such as those induced by obesity (Wende and Abel, 2010). By this mean, the present study intends to analyze by proteomic approach the acute effects of a single bout of moderate (90% of MLSS) and high (incremental workload test) exercise intensities on the heart proteome of obese (ob/ob) and non-obese mice (ob/OB).

Materials and Methods

Animal and group design

The experiments were conducted according to the regulations of the Ethics Committee for Animal Care (CEUA) of Biologic Sciences Institute of the University of Brasilia–Brazil, UnBDOC no. 29299/2009. Eighteen to 12-week-old C57BL/6J mice were divided into two groups according to mice phenotype: heterozygote ob/OB ($n = 9$) non-obese mice and leptin-deficient C57BL/6J homozygote ob/ob obese mice ($n = 9$). Each group was divided into three different treatments: control (C), moderate exercise (↑↓) and high exercise (↑). All animals received food and water ad libitum.

Intensity determination and exercise performance

Moderate and high swimming exercises were defined according to maximal lactate steady state threshold (MLSS), considered the gold standard in aerobic fitness assessment (Beneke et al., 2011). MLSS determination was in accordance with the protocol used by Almeida et al. (2011). Moderate intensity exercise session was performed by one bout of 20 min of swimming with an overload representing 90% of MLSS. High intensity exercise was performed by a single incremental workload test adapted by Cunha et al. (2009) and recently also previously performed in ob/ob mice by Almeida et al. (2011). Prior to the exercise session and aiming to minimize the animals stress without promoting physiological adaptations derived from exercise, all animals prepared for the single bout of exercise were submitted to water environment. All water activities were performed in a swimming tank containing pre-warmed water (30–32°C).

Euthanasia and tissue excision

Forty-five minutes after the single bout of exercise, each animal was fully anesthetized and euthanasiated by cervical dislocation. Heart and liver were excised, weighed immediately (Manter® AL500g, SP, Brazil), frozen on liquid nitrogen and kept at −80°C.

Protein extraction and quantification

Heart tissues were homogenized in 10 mM Tris–EDTA buffer pH 7.4 containing 250 mM sucrose and 2 mM EDTA. Homogenate was centrifuged at 1,000*g* for 10 min and precipitate was then collected and re-homogenized in 10 mM Tris–HCl buffer pH 7.2 containing 176 mM KCl and 2 mM EDTA and once again centrifuged at 1,000*g* for 10 min as described elsewhere (Short et al., 2005). Supernatant was then collected for protein quantification by fluorimetric method (Qubit® Invitrogen/Life Technologies Van Allen Way, Carlsbad, CA). Samples were adjusted in aliquots containing 30 μg for 1D electrophoretic analysis and 600 μg for 2-DE.

Electrophoretic analysis

A total of 600 μg of the tissue extract was precipitated using a 2D Clean-Up Kit (Amersham Biosciences/GE HealthCare, Uppsala, Sweden) and homogenized in a 266 μl solution of 2% CHAPS, 8 M urea, 2 M thiourea, 65 mM DTT and 1% IPG buffer for immobilized pH gradient 3–11 nonlinear strips. Homogenate was then applied to a 13 cm 3–11 nonlinear immobilized pH strip (General Electric) for isoelectric focusing. Strips were rehydrated in this solution for 16 h at 22°C constant temperature. Isoelectric focusing was performed on the Ettan™ IPGphor 3 equipment (General Electrics) programmed for gradient mode (30 min at 500 V, 90 min at 1,000 V and 90 min at 3,500 V, maintained at this voltage until strips had accumulated a minimum of 50.000 V h 50 mA was applied to each strip at a constant temperature of 20°C. After the first dimension, strips were equilibrated for 15 min with a 50 mM Tris–HCl pH 8.8 buffer containing 6 M urea, 30% glycerol, 2% SDS and 0.1% DTT. Second dimension was performed on 1.5 mm SDS–PAGE 12.5% gels. Electrophoresis was conducted on the Dalt-6 equipment (General Electric) at 600 V, 90 mA and 100 W for 30 min and then 700 V, 240 mA and 100 W for approximately 8 h. Afterward, gels were overnight stained with Coomassie blue G-250 (General Electrics).

In silico gel analysis

A total of 54 2-DE gels were run in order to attempt three technical replicates of each biological sample from all treatment groups (control; moderate and high exercise) and both animal phenotypes (ob/ob and ob/OB) (Fig. 1). All gels were screened on an HP scanner (Scanjet 8290) and afterwards analyzed by BioNumerics 5.0 (Applied Maths) software. Digitized gel images were all converted to tif files and a calibration curve was applied to convert all color tones into a gray scale (16-bit, 600 dpi). All technical replicates were aligned and screened by the software in order to identify the same vectors. By this procedure, spots were located and analyzed by their molecular mass and isoelectric point position guided by the selected vectors. To search for horologes spots in different gels, Bionumerics software uses a reference system based on a collection of reference spots termed as landmarks with equivalent coordination around different areas of the gel as well as, mass and isoelectric point in different experimental gels. Once the reference gel system is created, other gels are normalized by automatically matching the selected spots to the landmark spots in the reference system. Spots screening was performed by automated software analysis followed by visual inspection from tree independent proteome analysis experts. To avoid spots misidentification, spots were only considered for further analysis if it were identified in at least two out of three gel replicates, otherwise it were considered as an artifact been not considered for analysis. Aiming to guarantee technical reproducibility and similarities between 2-DE experiments a correlation analysis (R^2) was applied across all 2-DE images between technical replicas from every biologic sample. Detected spots were correlated by linear regression performed by Image Master 2D Platinum v7.0™. Mean value of spot volume (dot per inch) from all three replicate spot densities was used for further protein modulation comparison between all normalized volumes of the other treatments. For spots selection additional criterion was utilized. Only spots with relative volume equal to or bigger than 0.1 dpi were selected (Maria-Neto et al., 2012; Tonietto et al., 2012). For comparison, remaining spots had to present at least a delta (Δ) fold change of two units of intensity between any one of the treatments (Tonietto et al., 2012). All spots included by these two criteria were classified as down or up-folded when compared to control treatment.

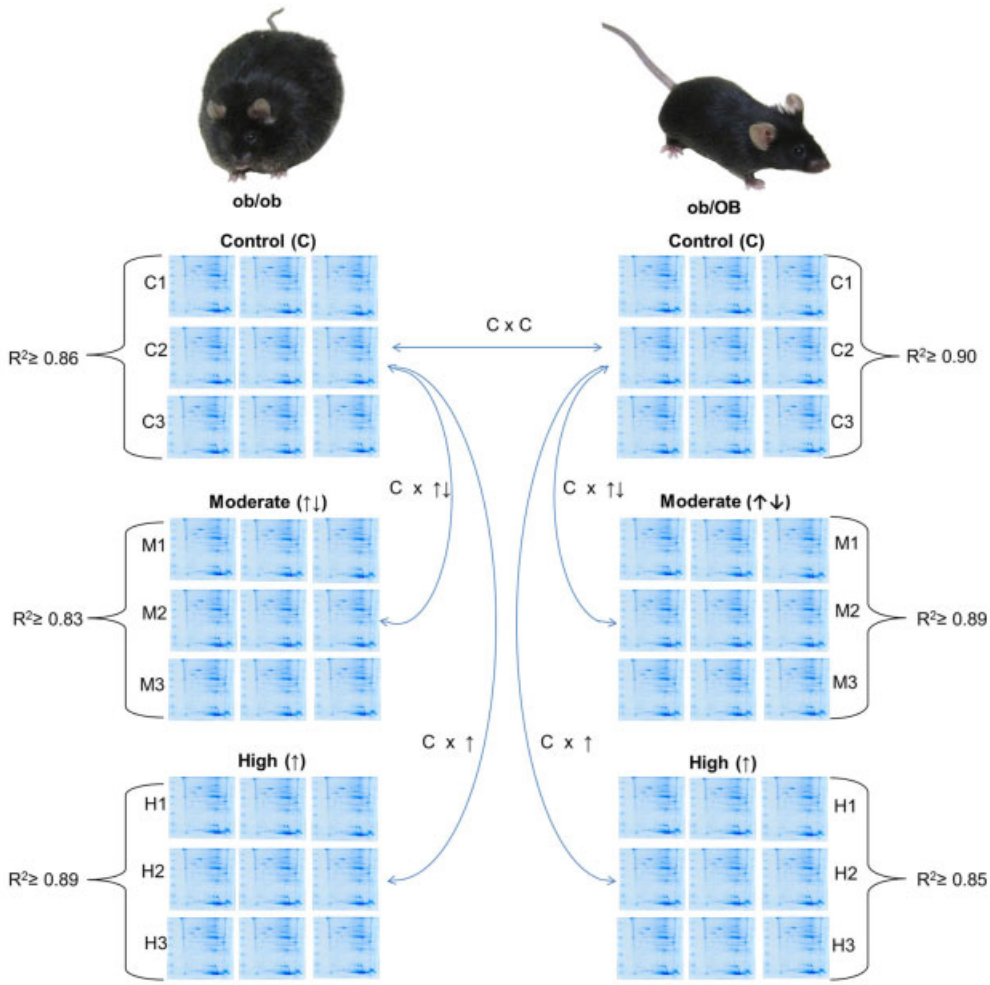

Fig. 1. Gel analysis design. Each one of the five gels from all treatments was cross linked for technical reproducibility analysis. Mean values of spots' relative volume from each group were cross linked between each treatment (C × ↑↓; C × ↑) in order to verify the acute modulations of both exercise intensities on obese (ob/ob) and non-obese (ob/OB) heart and the differences between the two intensities. Control treatments (C × C) from ob/ob and ob/OB mice were crossed in order to verify obesity modulation in the heart.

Protein identification by mass spectrometry

Selected spots were excised from gels (~2–3 mm peace of diameter) using a scalpel. Up to three spots from the three gel replicate were placed together in a 1.5 μl tube. Excised spots were washed with 400 μl of 50% acetonitrile, 25 mM NH_4HCO_3 for 20 min on vortex and then the solution was replaced with acetonitrile at 100% for 10 min. Spots were dried in a SpeedVac for 20 min. Protein in-gel digestion was carried out with Sequencing Grade Modified Trypsin (Promega, Madison, WI) according to (Shevchenko et al., 2006) with modifications. An amount of 650 ng of trypsin was diluted in 20 μl of 50 mM acetic acid buffer (32.5 ng ml^{-1}) containing 180 μl of 50 mM NH_4CO_3. All spots with trypsin were incubated on ice for 30 min. Afterwards 40 μl of 50 mM NH_4CO_3 was added to each tube and all were incubated for 22 h at a constant temperature of 37°C. The peptides derived from tryptic digestion were analyzed using an UltraFlex II MALDI-TOF–TOF (Matrix-Assisted Laser Desorption Ionization Time-of-Flight, Bruker Daltonics, Billerica, MA). A sample of 2 μl was mixed in 6 μl of 0.1% (α-cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid and acetonitrile (1:1). A volume of 0.5 μl was applied to a MALDI-TOF plate and air-dried at room temperature. Spectrometry was operated in a linear mode for MS acquisition and reflected mode for MS/MS acquisitions using modulated power with 200 random shoots. Spots were identified using peptide mass fingerprinting (PMF). The mass list for each sample was analyzed using the program MASCOT v2.1.0, Matrix Science, London (http://www.matrixscience.com) assuming one missed cleavage, carboxymethylation and methionine oxidation as modification. The list of masses was compared against the non-redundant *Mus musculus* NCBI database (Kernec et al., 2001; Gazzana and Borlak, 2009). MASCOT analysis set a significance level at $P < 0.05$, the obtained MS data indicates that the score leaded by peptide mass fingerprinting provides the statistically most probable protein identification. Results were evaluated by comparing the molecular mass and isoelectric point of the most scored hit with data observed in 2-DE gels.

Statistical analysis

Heart proteome modulation by obesity or exercise was evaluated by comparing mean values of 2-DE spots relative volume. Values are expressed as means ± SD. All intensity data were standardized before further analysis. The proteomics intensity data were tested

for normality (Shapiro–Wilk), and equal variance (*t*-test for homogeneity of variance for two dependent samples), and if data were normally distributed, the significance of treatment was determined by one-way analysis of variance (ANOVA) followed by Tukey's test. GraphPad prism software v. 5.0 was used for both statistical analysis and graphical presentation in all studies.

Results

Physiologic parameter of experimental animals

The general physiologic parameters of heterozygous ob/OB (n = 9) and homozygous leptin-deficient (ob/ob) obese mice (n = 9) are shown in Table 1. In contrast to ob/ob obese mice, ob/OB non-obese mice were used as controls to evaluate the effect of obesity and exercise on mice hearts. As expected, body and liver weight from (ob/ob) obese mice were significantly heavier than (ob/OB) mice ($P < 0.05$) (Table 1). The ob/ob mice hearts weights are shown also to be increased in comparison to non-obese ob/OB mice ($P < 0.05$). Maximal lactate steady state (MLSS) was established corresponding at intensities of 2.4% of body weight (BW) for ob/OB mice and by 4.6% BW for ob/ob mice ($P < 0.05$). Animals performed incremental test (IT) representing high exercise at the maximal workload corresponding to 2.7 ± 01.2% BW for ob/OB mice and 4.2 ± 0.5% BW for ob/ob mice ($P < 0.05$) (Table 1).

General proteomic analysis

Our proteomic experimental results were divided into two sections. First the acute effects of moderate and high exercise intensity on obese (ob/ob) and non-obese (ob/OB) mice heart proteome and second, the effect of 12 weeks of leptin knockout-obesity on heart proteome. Correlation mean values (R^2) from 2-DE experiments are presented in Figure 1. Software screening counted mean values of 150 and 131 spots respectively from ob/OB and ob/ob 2-DE gels (Table 2). Most of spots were resolved approximately in the 25–90 kDa molecular mass range and by the 6.0–9 pH area. The inclusion criteria (spot relative volume ≥0.1 dpi and delta (Δ) of two units of fold change between any one of the treatments or mice groups) have considered a total of 33 spots for mass spectrometry analysis. Of those, 12 spots were identified by MS/MS or PMF as indicated by arrow in Figure 2 and described in Table 3. Statistical analysis ($P \leq 0.05$) has confirmed nine proteins to be significantly modulated by exercise (n = 7) or obesity (n = 2) when compared to non-exercised and non-obesity control groups. These 12 spots were categorized in four different groups by their biological function according to UniProt domain (www.uniprot.org), as predominantly inherent to transport (17%) and metabolic group (41%) followed by contractile-cytoskeleton (17%), signaling (17%) and unknown function (8%) (Fig. 3).

Acute effects of moderate and high exercise in heart proteome modulation

Exercise training is well known to promote cardiovascular adaptation and cardio protection been the modulation of heart proteome an essential part of this process (Burniston and Hoffman, 2011). As expected moderate and high exercise intensities promoted acute proteome modulation on cardiac tissue. By this way, the present study showed seven spots with differential expression followed by exercise stimulus. After a single bout of moderate or high swimming exercise, aspartate aminotransferase (1.9-fold $P \leq 0.05$ by high exercise in ob/OB mice—Fig. 4D) and zinc finger protein (2.1- and 2.4-folds $P \leq 0.05$ after moderate and high exercise in ob/OB mice—Fig. 4F) were up-regulated in ob/OB mice, followed by myoglobin (Mb) up-regulation (1.5 and 1.5 $P \leq 0.05$ after moderate and high exercise) in ob/ob mice (Fig. 4). Thus, nucleoside kinase diphosphate B (NDK-B) was down-regulated (1.8-fold $P \leq 0.05$ by high exercise in ob/OB mice—Fig. 4E) on ob/OB mice. Mitochondrial aconitase was seen to be down-regulated in both animal groups (1.8-fold $P \leq 0.05$ by high exercise in ob/OB and 2.7-fold by moderate and 3.1-fold ($P \leq 0.05$) by high exercise in ob/ob mice—Fig. 4B,C). Heart–fatty acid-binding protein was also shown to be down-regulated in ob/ob mice (1.61-fold $P \leq 0.05$ by high exercise—Fig. 4G).

TABLE 2. Mean values of spots detected on every treatment and mice group by Bionumerics® software

Group	Treatments	Means ± SD
ob/OB	C	146 ± 6
	↑↓	140 ± 6
	↑	165 ± 4
ob/ob	C	125 ± 6
	↑↓	125 ± 9
	↑	145 ± 9

C, control group; ↑↓, moderate intensity; ↑, high intensity.

Effects of obesity in heart proteome

Obesity induced by leptin deficiency in ob/ob mice is known to promote lipotoxicity in cardiac myocyte leading to heart dysfunction. Proteomic 2-DE tool was used to verify these modulations within obese heart (ob/ob) compared to non-obese heart (ob/OB). Twelve weeks of obesity-induced by leptin knockout was observed by us to promote the modulation of two proteins (α-actin and zinc finger proteins) showing differential expression between the obese (ob/ob) and non-obese (ob/OB) control hearts. Both proteins were up-regulated (heart α-actin; 1.9-fold $P \leq 0.05$ and zinc finger protein; threefold increase $P \leq 0.05$) respectively shown on Figure 5A,B.

Discussion

Exercise is known to improve myocardium phenotype and attenuate pathological heart modulations (Kolwicz et al., 2009). However, the wide range of exercise stimulus, including intensity, duration and nature still needs more research to understand specific molecular pathways and their improvement that lead to cardioprotection. The initial molecular modulation in response to a single bout of exercise may provide further understanding on how the maladaptive phenotype is reduced leading to a positive adaptation. Trying to answer these

TABLE 1. General physiologic parameters of 12-week-old heterozygous (ob/OB) and homozygous ob/ob obese mice

Mouse group	Body weight (g)	Heart weight (g)	Liver weight (g)	MLSS[a] (% BW)	90% MLSS[a] (% BW)	IT workload[b] (% BW)
ob/OB (n = 9)	29.6 ± 3.9	0.206 ± 0.03	1.793 ± 2.26	2.4 ± 0.5**	2.2 ± 0.3**	2.7 ± 1.2**
ob/ob (n = 9)	57.5 ± 11.5*	0.244 ± 0.01*	3.361 ± 0.66*	4.6 ± 0.5	4.2 ± 0.4	4.2 ± 0.5

Body, heart and liver weight data are means ± SD.
[a]Only moderate exercise group (n = 6).
[b]Only incremental workload test group (n = 6); BW, body weight.
*$P < 0.05$ versus ob/OB; MLSS, 90%MLSS and IT workload data are means ± SD.
**$P < 0.05$ versus ob/ob.

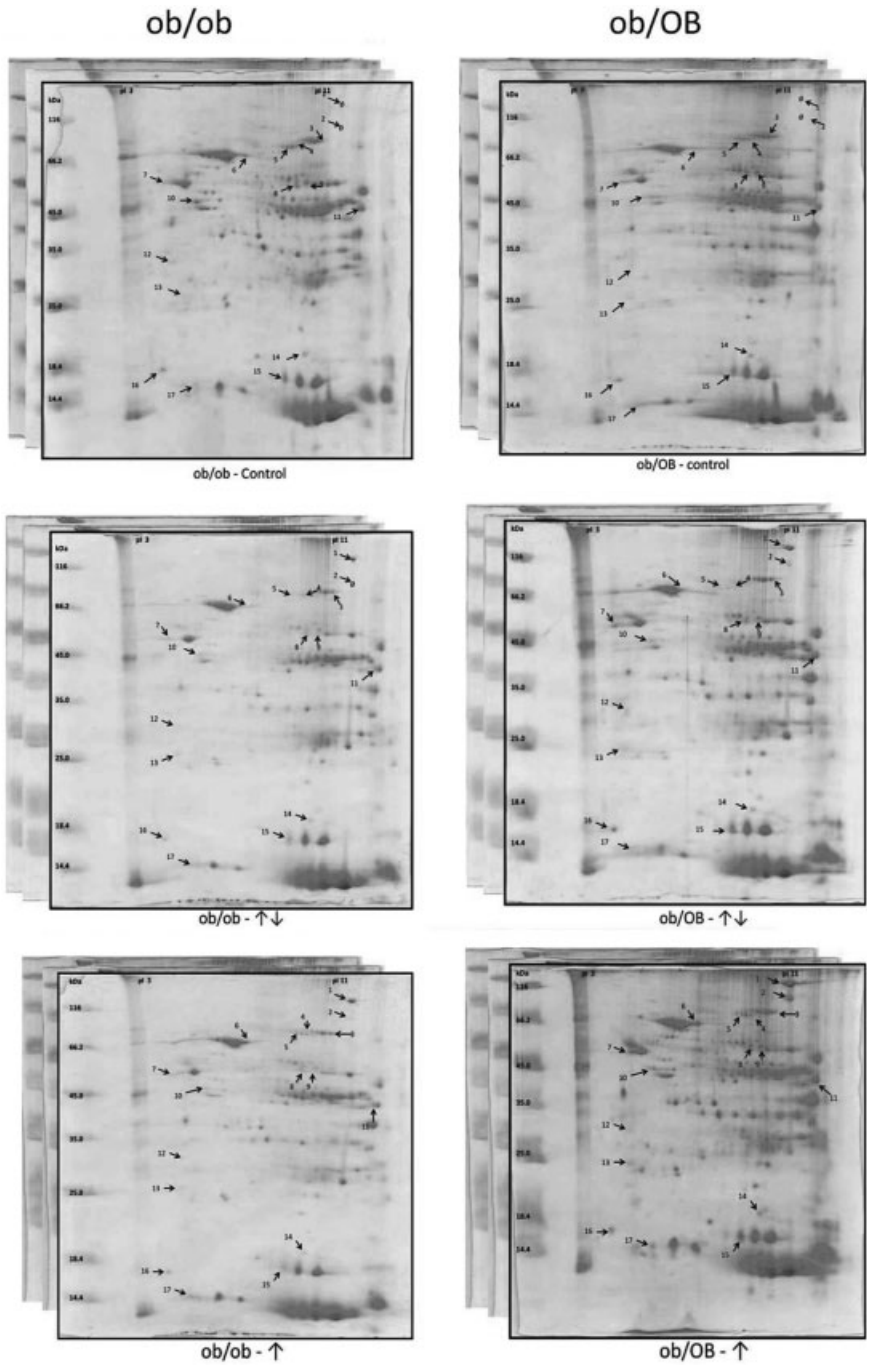

Fig. 2. 2-DE gels from control (C), moderate (↑↓) and high (↑) exercise intensities from ob/OB (non-obese) and ob/ob (obese mice). Each analysis conducted with three technical replicates. Arrows indicate protein spots identified by peptide mass fingerprint (PMF) or MS/MS.

questions, 2-DE proteomic analysis was used to catalog and understand the exercise-induced changes in the obese and non-obese heart and thus verify possible new molecular candidates for heart improvement by exercise.

Leptin-deficient ob/ob mice are characterized by the knockout of the *ob* gene, responsible for encoding the adipose derived hormone–protein leptin attributed to food intake, satiety control and energy expenditure. These mice rapidly assume an obese phenotype as well as obesity-associated pathologic complications, such as hyperglycemia, hyperinsulinemia, type 2 diabetes and cardiomyocyte dysfunction (Breslow et al., 1999). As expected, after 12 weeks of life, body weight was significantly higher in ob/ob mice compared to non-obese ob/OB mice ($P < 0.05$) (Table 1) as previously shown (Breslow et al., 1999). Difference in body weight is a direct response to leptin knockout, leading to metabolic homeostasis dysfunction and obesity (Zhang et al., 1994). Throughout body weight upgrade, ob/ob hearts are

TABLE 3. List of protein identified by peptide mass fingerprint (PMF) and MS/MS

No.	Protein	NCBI protein ID	Function	pI/Mr (kDa)	MASCOT score/coverage (%)	Identification	Peptide sequence
3	Aconitate hydratase, mitochondrial precursor-2	NP_542364	Catalyzes the process of citrate to isocitrate	8.08/86161	96/21	PMF	
7	Mitochondrial ATP synthase, H^+ transporting F1 complex beta subunit	ABD77233	Mitochondrial membrane	4.90/48047	141/37	PMF	
8	Zinc finger protein-2	NP_001038162	Transcriptional factor	9.10/54154	71/21	PMF	
9	ATP synthase subunit alpha, mitochondrial precursor	NP_031531	Mitochondrial membrane	9.22/59830	79/27	PMF	
10	Alpha-cardiac actin	CAA27397/AAA37167	Myofibrilar protein	5.45/38016 5.23/42043	42/2 42/2	MS/MS	R.GYSFVTTAER.E
11	Mitochondrial aspartate aminotransferase	AAA37265	Amino acid metabolism enzyme	9.05/48123	74/3	MS/MS	R.FVTVQTISGTGALR.V
12	Myosin light chain 3	NP_034989	Myosin regulatory chain	5.03/22521	72/26	PMF	
13	Translationally-controlled tumor protein	NP_033455	Signaling factor	4.76/19564	131/8	MS/MS	R.DLISHDELFSDIYK.I
14	Nucleoside diphosphate kinase B	NP_032731	Nucleoside synthesis	6.97/17466	44/6	MS/MS	K.DRPFFPGLVK.Y
15	Myoglobin—Mb	NP_598738	Intramuscular oxygen binding protein	6.94/17116	199/11	PMF MS/MS	K.VEADLAGHGQEVLIGLFK.T
16	Unnamed protein product	CAA24214		5.23/11645	60/1	MS/MS	R.LENEIQTYR.S
17	Heart–fatty acid-binding protein	NP_034304	Intracellular fatty acid binding protein	6.11/14810	78/15	MS/MS	NTEINFQLGIEFDEVTADDR

Score; statistically significant ($P < 0.05$) values by MASCOT software according to a MOUSE (*Mus musculus*) peptide and protein NCBI database.

characterized by their hypertrophy phenotype as shown by Dong et al. (2006) after 12 weeks of age (ob/ob; 0.33 ± 0.01 g vs. non-obese; 0.20 ± 0.01 g).

The significantly higher values of % body weight verified in MLSS (ob/OB 4.6 ± 0.5 vs. ob/ob 2.4 ± 0.5, $P \leq 0.05$) and IT (ob/OB 2.7 ± 1.2 vs. ob/ob 4.2 ± 0.5, $P \leq 0.05$) of ob/ob compared to ob/OB may be resultant by the greater volume of adipose tissue of the homozygote mice, which may facilitate animal fluctuation during exercise. This hypothesis has also been discussed by Almeida et al. (2011) that have reported similar data. These authors indicated that the higher MLSS %BW from ob/ob compared to ob/OB mice does not indicate a higher aerobic capacity, been a consequence of the higher body fat percentage.

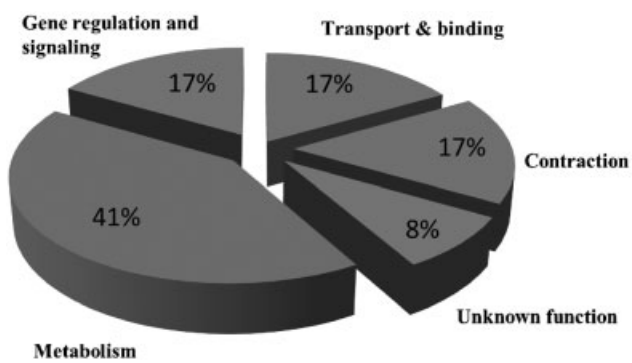

Fig. 3. Protein functions Protein predicted function according to UniProt domain (http://www.uniprot.org).

Effect of exercise on heart proteome

Previous heart proteome studies have shown similar proteome data, ranging from 13 to 26 differential protein spots in response to exercise. These studies were conducted on different heart portions and specific organelles, such as the left ventricle, left ventricle free wall and mitochondria sub-populations as reviewed by Burniston and Hoffman (2011). Some of the research was conducted by pathological insults, such as myocardial infarction (Bansal et al., 2010), ischemia (Kavazis et al., 2009) or by a variety of exercise stimulus, such as short and long moderate endurance or high intensity training (Petriz et al., 2011). Despite the variety on scope and methodology, all of these studies were conducted by chronic exercise stimulus. Therefore our research is the first to evaluate acute proteomic modulation after one single bout from two different exercise intensities using leptin-deficient obese and non-obese mouse hearts.

Firstly, the down-regulation of mitochondrial aconitase (1.8-fold by high exercise in ob/OB and 2.7-fold by moderate and 3.1-fold by high exercise in ob/ob mice—Fig. 4B,C); NDK-B (1.8-fold by high exercise in ob/OB mice—Fig. 4E) and FABP (1.6-fold by high exercise in ob/ob mice—Figure 4G) and the up-regulation of aspartate aminotransferase (1.9-fold by high exercise in ob/OB mice—Fig. 4D) demonstrates the sensibility of heart bioenergetics enzymes to one single session of moderate or high exercise. Mitochondrial aconitase data shown here are similar to other research results, demonstrating its down-regulation by different exercise stimuli such as, a 6-week (70–75% VO_2 peak) endurance program (Burniston, 2009) and an 8-week moderate (55–60% VO_2 max) program, followed by myocardial infarction (Bansal et al., 2010). Inherent to the tricarboxylic acid cycle, (TCA), this enzyme converts citrate into isocitrate in one of the primary TCA steps, been its response to exercise by a proteomic analysis well described by Burniston (2008) which demonstrated its down-regulation in skeletal muscle after moderate (70–75% VO_2 peak) endurance

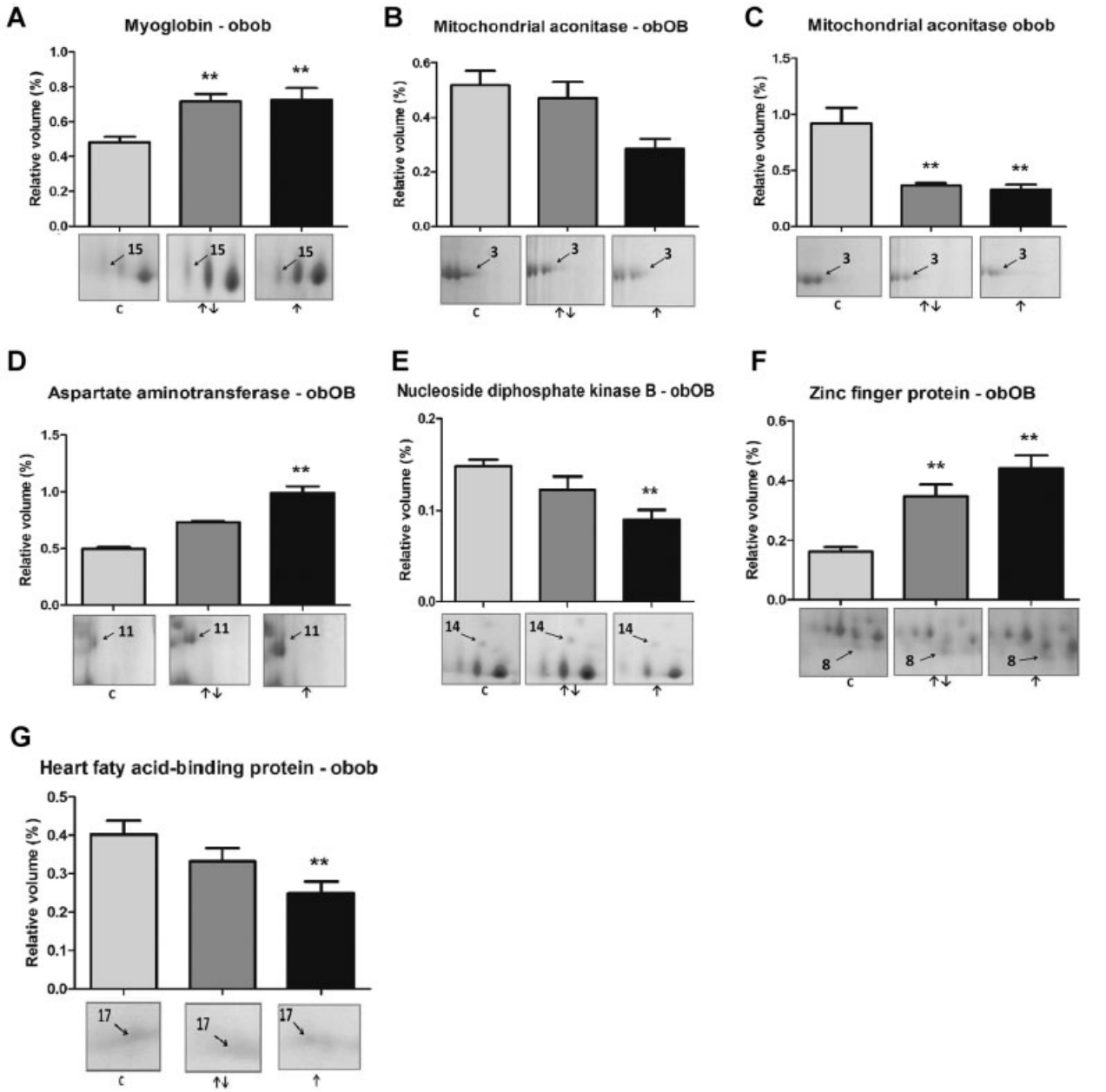

Fig. 4. Exercise histograms of protein expression profiles Histograms of protein expression of control (C), moderate (↑↓) and high (↑) exercise intensities on ob/OB and ob/ob mice heart. The effect of exercise on transporting and binding proteins: Mb (A: ob/ob). Metabolic proteins: Aconitase (B: ob/OB; C: ob/ob), Aspt (D: ob/OB), NDK-B (E: ob/OB) and FABP (G: ob/ob). Signaling proteins: Zfp (F: ob/OB). Data are means ± SD. $^{**}P < 0.05$ versus control (C).

exercise. Aconitase is highly suitable to oxidative stress (Tortora et al., 2007), which leads this enzyme to carbonylation as a response to reactive oxygen species and its loss of biologic function (Levine, 2002). Aconitase down-regulation may occur as a response to chronic oxidative exercise stress (Burniston, 2009; Bansal et al., 2010) as well to acute exercise (our data) disturbing mitochondrial energy production. Considering aconitase's similar reduction after moderate and high exercise in the obese group (Fig. 4C) may indicate that leptin-obesity phenotype is more suitable to exercise oxidative stress. The fact that aconitase is down-regulated immediately after exercise (our data) and also by 4 h after chronic exercise (Burniston, 2009), suggests a similar enzyme profile towards different exercise stimuli. A similar down-regulation has also been seen after chronic exercise in rat afterwards myocardial infarction (Bansal et al., 2010). Despite of Bansal et al. (2010), data and results here presented using ob/ob mice, further analysis are necessary to elucidate the role of exercise on this metabolic enzyme in obesity or myocardial dysfunctions.

The amino acid metabolic enzyme, aspartate aminotransferase (Aspt), was also identified being up-regulated after high (1.9-fold, $P \leq 0.05$) exercise in non-obese ob/OB mice (Fig. 4D). This enzyme also known as aspartate transaminase, catalyzes the process of α-amino group transport from

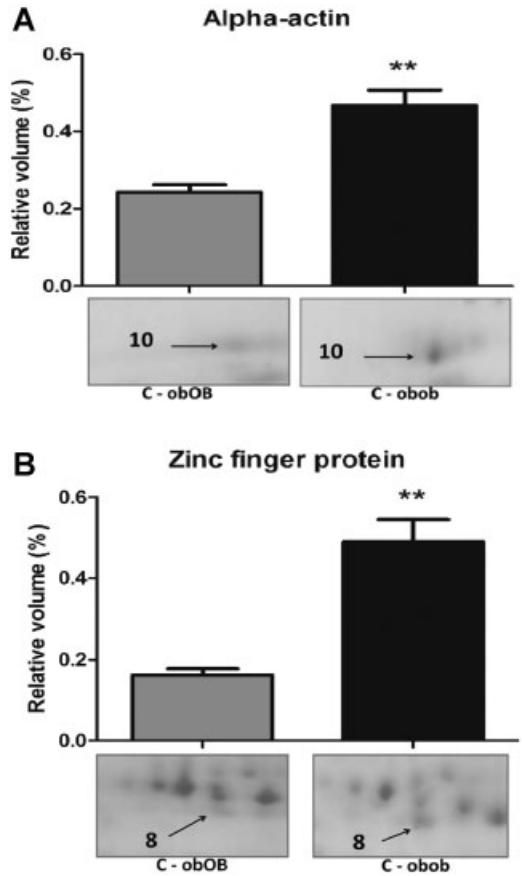

Fig. 5. Obesity histograms of protein expression profiles. Histogram of protein expression of control (C) treatment from ob/OB and ob/ob mice in order to elucidate obesity effect on heart proteome. Contractile protein: α-actin (A) and signaling protein: Zfp (B). Data are means ± SD. $^{**}P < 0.05$ versus control (C) ob/OB.

aspartate to glutamate, being a biomarker for skeletal and cardiac muscle damage. Recently it has been show that serum Aspt was elevated up to 24 h after an endurance running training in human (Nie et al., 2011). Similar to our data, other proteomic research has also shown Aspt up-regulation after chronic exercise (Burniston, 2009) after ischemic-exercise insult (Kavazis et al., 2009).

Our study has also identified the heart fatty acid binding protein (FABP), showing a decrease of 1.6-fold ($P \leq 0.05$) after high exercise compared to control in obese mice (Fig. 4G). These 13–15 kDa range proteins are abundant in tissues with high rates of fatty acid metabolism and oxidation, such as the myocardium, which suggest the major importance of this protein in heart during exercise. It is suggested that these proteins facilitate fatty acid uptake into cell membrane and its intracellular transport to mitochondria, confirming its important role in cellular FFA homeostasis (Shearer et al., 2005). Therefore, the role and profile of FABP through exercise stimulus has not yet been fully elucidated. Research indicates different responses of FABP by exercise, such as its up-regulation after chronic exercise (Burniston, 2009), as well as its non-response to 8 weeks of endurance training (Clavel et al., 2002). Our data also indicated a non-response of FABP in non-obese (ob/OB) mice after moderate or high acute exercise. Until now there there's no proteomic data demonstrating FABP behavior in transgenic obese (ob/ob) mice submitted to exercise. Considering its important participation in cellular FFA homeostasis and its possible major role in cardiac metabolism through exercise, we suggest further research on FABP modulation by exercise stimulus.

Along with these findings, other metabolic enzymes such as mitochondrial ATP synthase (H^+ transporting F1 complex beta subunit) and ATP synthase (subunit alpha) have been identified (Table 2 and Fig. 2, arrows 7 and 9). Even that proteomic research has confirmed the effect of exercise over several mitochondria proteins in heart (Petriz et al., 2011), these enzymes showed no acute response to our proposed exercise treatment in both animals groups.

Being inherent to muscle as its main oxygen stock, myoglobin (Mb) was shown to be up-regulated similarly by moderate (1.48-fold $P \leq 0.05$) and high (1.5-fold $P \leq 0.05$) exercise in ob/ob but not in ob/OB mice (Fig. 4A). Our data demonstrate an acute exercise response, also verified by Lippi et al. (2008) that observed an acute myoglobin up-regulation after an endurance running. Myoglobin synthesis responds to a single session of moderate and high exercise, possibly indicating initial metabolic efforts of obese mice's myocardium to reach oxygen homeostasis. Therefore, Mb is a well-known marker of muscle injury, highly associated to exercise-induced muscle damage been its levels acutely elevated followed by endurance exercise (Lippi et al., 2008). Thus, the enhanced amount of Mb after moderate and high intensities may reflect initial signaling of muscle damage.

Two proteins inherent to the contractile process were here identified as cardiac α-actin and myosin light polypeptide chain-3 (Table 2 and Fig. 2; arrows 10 and 12). As expected, data here indicated that none of these proteins was modulated by a single bout of moderate or high exercise. The up-regulation of myofibrilar proteins is, moreover, associated with chronic exercise usually leading to adaptive (Burniston, 2009) or maladaptive hypertrophy in myocardium (Sun et al., 2008).

Despite myofibril data, exercise has been shown to up-regulate zinc finger protein (2.1- and 2.4-folds $P \leq 0.05$ after moderate and high exercise in ob/OB mice—Fig. 4F). As a transcriptional factor, zinc finger proteins (Zfp) are expressed in several organisms, playing a role in many physiological processes, such as cellular differentiation, proliferation and apoptosis (Pieler and Bellefroid, 1994). Research has evidenced that Zfp transcriptional levels are up-regulated by many stressor stimuli and that this up-regulation could be associated with oxidative stress vulnerability (Nogusa et al., 2006). As a stress agent, acute and chronic exercise is well known to promote oxidative stress (Fisher-Wellman and Bloomer, 2009), which may explain Zfp up-regulation. However, a gene encoding a zinc finger type has been seen to be down-regulated after 150 min of swimming by trained mice compared to sedentary mice (Takahashi and Kubota, 2005). The zinc finger-like domain has been described as assisting heat shock protein 70 in chaperoning and protein folding (Lu and Cyr, 1998), which may possibly indicate cellular homeostasis assistance after stress insults, such as exercise. All of these divergent protein responses from pathologic (obesity) and non-pathologic hearts, after exercise stimulus, may indicate their peculiar ability to molecular signaling. However there are few data into the role of Zfp through exercise stimulus, leading any conclusion about its profile during or after exercise premature.

Figure 6A presents an overview of the identified proteins, acutely modulated by exercise in cardiomyocytes. As previously discussed, moderate or high exercise effected mitochondrial aconitase, heart fatty acid binding protein and nucleoside diphosphate kinase B leading these proteins to be down-regulated at protein level compared to control non-exercised heart. Being aconitase essentially linked to carbohydrate metabolism and H-FABP to fatty acid transport to

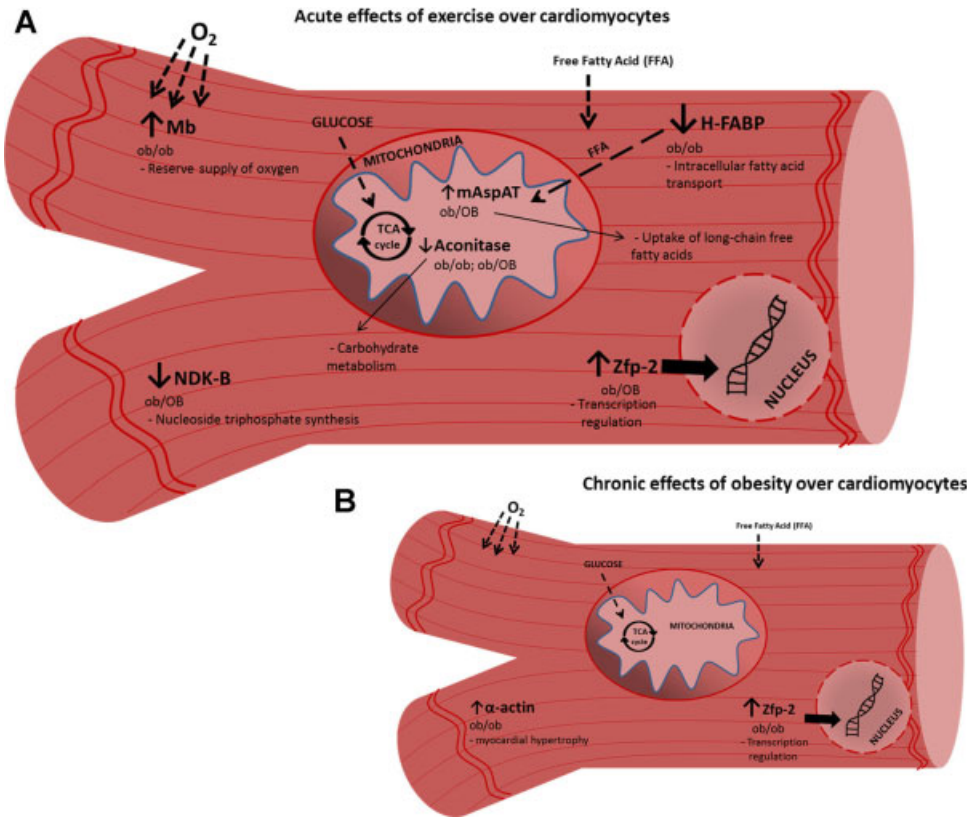

Fig. 6. Exercise and obesity effects over identified cardiomyocyte proteome Overview of the acute effects of exercise (up- (↑) or down- (↓) regulation) over the identified protein from obese (ob/ob) and non-obese (ob/OB) cardiomyocyte (A). Letter (B) represents the effect of chronic obesity over α-actin and Zfp in obese (ob/ob) cardiomyocytes compared to non-obese (ob/OB) group.

mitochondrial and its oxidation, as well as NDK-B to nucleosides triphosphate synthesis, it can be seen that, these bioenergetics molecules are acutely affected after a single session of exercise possibly until restoring of cell homeostasis. The rapid up-regulation of myoglobin after exercise bout is also an indication of acute response to cardiomyocyte demand of oxygen protein storing, possibly more relevant in obese (ob/ob) than in non-obese heart. As a cytosolic component, aspartate aminotransferase is attributed to tissue damage associated to exercise overload. Therefore, its isoform here identified, perform long chain free fatty acid uptake within mitochondria, been highly related to the process of FFA oxidation. The up-regulation on non-obese heart not seen in obese heart may indicate a possible impairment in the process of fatty acid oxidation in obese heart which is one of the mechanisms of heart lipotoxicity (Abel et al., 2008). The several functions related to zinc finger protein as a signaling molecule with translation regulation function lead its up-regulation to indicate exercise as and extrinsic agent that interferes within the transcriptional process, however, the magnitude and the exact effects of this protein modulation by exercise still not elucidated.

Effects of obesity in heart proteome

To date, our study is according to our knowledge the first to analyze the effects of obesity modulation on leptin-deficient ob/ob mice heart proteome. Up-regulated in the ob/ob hearts by 1.9-fold (Fig. 5A), cardiac α-actin is one of the actin isoforms reported as predominant in the adult mouse heart. However, it is known that the rodent embryonic and fetal heart presents a co-expression of cardiac and skeletal α-actin genes (Vandekerckhove et al., 1986). Playing a key role in cell motility and contractility, α-actin could be associated with myocardial hypertrophy derived from exercise and by maladaptive hypertrophy as a cardiomyopathy phenotype (Mogensen et al., 1999; Lim et al., 2001). The maladaptive process is, however, more associated with skeletal α-actin up-regulation in the heart (Lim et al., 2001). Our results demonstrate that exercise did not modulate cardiac α-actin by one single bout of moderate and high exercise, but protein expression was up-regulated (1.9-fold $P \leq 0.05$) in obese mice hearts compared to non-obese. Heart remodeling by obesity is resultant by a network of molecular modulations that can be accelerated or aggravated by other pathologic conditions such as metabolic and vascular disease (Abel et al., 2008). Some of these molecular modulations are a response to chronic delivery of high-levels of serum FFA, ectopic fat depots and infiltration and up-regulated contractile proteins (shifts of myosin α and β-heavy chain proportion, collagen accumulation), leading to heart metabolic and contractile impairment (Abel et al., 2008; Wende and Abel, 2010). Since cardiac α-actin gene has been associated with hypertrophic cardiomyopathy (Mogensen et al., 1999), its response to chronic obesity development with a 1.9-fold increase (Fig. 5A) may indicate molecular signaling towards further maladaptive hypertrophy. Besides the cardiac α-actin protein up-regulation here showed, cardiomyopathy has been associated with high levels of skeletal α-actin (Lim et al., 2001).

Since it is highly similar (98% identity) and co-expressed in the mouse heart, it is possible that skeletal α-actin may have also been expressed within cardiac α-actin, as discussed by Boluyt et al. [2006].

The 12 weeks of obesity development by leptin deficiency may be seen as a chronic stressor stimulus leading to oxidative stress on the myocardium (Furukawa et al., 2004). Similar to its acute response to exercise, Zfp's threefold increase (Fig. 5B) may indicate that this protein responds to chronic stimulus as well as to 12 weeks of obesity. These data enforce the idea of Zfp sensitivity to stressor agents and its response to oxidative stress as previously described. A novel gene with zinc finger activity MCPIP (monocyte chemoattractant protein–induced protein) was shown by microarray analysis to stimulate many genes related to cell apoptosis and death in cardiac myoblast (Younce and Kolattukudy, 2010). The same authors demonstrated cardiomyocyte cell death by hyperglycemia linked to these zinc finger proteins (Younce et al., 2010), as well as its role in adipogenesis induction (Younce et al., 2009). These data demonstrate a close relationship between Zfp and some pathologic processes leading to heart impairment. Therefore, more research may be necessary to verify the role of the transcriptional factor protein toward lipotoxicity within the heart.

Zinc finger protein and α-actin are both shown on Figure 6B as their response to chronic obesity influence within the heart tissue. Obviously these two proteins do not represent the entire core of proteins that are modulated by obesity in the heart; however, the significant up-regulation of these proteins confirm some literature findings such as maladaptive cardiomyocyte hypertrophy by α-actin up-regulation. As exercise, obesity seems to signal towards transcription regulation by modulating zinc finger protein, however, as in exercise, the exact effects of this molecule modulation is still unclear in obesity and exercise.

One of the limitations of this study is the sample size per treatment ($n = 3$). One important reason for the reduced number of animals is the difficulty to obtain ob/ob mice, once these leptin-deficient mice are infertile. Thus its necessary the use of heterozygote animals for breeding leading to an time consuming and final pups of 75% of lean and 25% of obese mice (Almeida et al., 2011). The reduced number of ob/ob obese pups leads to a difficulty to obtain higher isogenic sample size. The sample size per treatment ($n = 3$) is not the most appropriate number to conduct an in vivo experiment. In this type of experimental arrangement ($P = 0.1$), is unable to reject the null hypothesis. Results achieved at this level of significance cannot be a reason to draw a conclusion about a population, however may be sufficient grounds for further study this phenomenon.

Conclusion

Here, the first analyses of the acute effects of a single bout of moderate and high exercise on leptin-deficient obese mice within its heart proteome were reported. These findings point out that both exercise intensities lead to acute proteome remodeling for metabolic (aconitase, Aspt, NDK-B), transporting (FABP) and signaling proteins (Zfp) on ob/ob and ob/OB mice hearts as shown on Figure 6A. To gain a better understanding of the molecular signaling that leads to heart impairment by obesity, we verified that contractile (α-cardiac actin) and translational factor protein (Zfp) were both up-regulated in the obese mice heart. These data demonstrate metabolic modulation towards obesity development by which some proteins, such as α-actin and Zfp are associated with maladaptive heart remodeling (Fig. 6B). Extensive and further molecular analyses are necessary to identity and better understand the vast molecular signaling inherent to metabolic and contractile impairment in the obesity-characterized heart. Key molecular identification may also bring insights into the mechanisms by which exercise leads to pathologic attenuation.

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