UNIVERSITY OF CALIFORNIA, SAN DIEGO

Changes in Mitochondrial Dynamics with Acute Single Bout Exercise

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Nareg Yeghia Kalajian

Committee in charge:

Professor Simon Schenk, Chair
Professor Michael David, Co-Chair
Professor Andrea Hevener
Professor Immo E. Scheffler

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The Thesis of Nareg Yeghia Kalajian is approved, and it is acceptable in quality and form for publication in microfilm and electronically:

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Co-Chair

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Chair

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ABSTRACT OF THE THESIS

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Nareg Yeghia Kalajian

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Metabolic syndrome (MetSyn) is a term used to describe risk factors associated with the increased risk of chronic diseases including cardiovascular disease, type 2 diabetes (T2DM), and certain forms of cancer. Exercise and caloric restriction have been shown to ameliorate features of the MetSyn, in part by improving insulin sensitivity and overall metabolic function. Improved metabolism and insulin action is thought to occur in response to enhanced

mitochondrial functional capacity, number, and mtDNA integrity. Surprisingly, despite the recent boom of mitochondrial dynamics and metabolic research, little is still known about exercise-induced alterations to mitochondrial dynamics. In this study I sought to characterize changes in expression and signaling of proteins involved in mitochondrial dynamics in response to acute exercise. Wild type C57Bl6/J female mice (n=24) were exercised on a treadmill with various durations. Quadriceps muscles were collected and flash frozen for subsequent analysis by PCR and immunoblotting. Acute single bout exercise led to increased Fis1 gene expression and protein levels. Mff protein levels on the mitochondria were increased with prolonged exercise. A novel and interesting finding was the increase of Drp1^{Ser616} phosphorylation in the 90-minute exercise group. This work provides novel and essential information on the exact mechanisms of mitochondrial fission. Now, more research on the recruiter proteins of the mitochondrial membranes will be able to build upon the discoveries of this thesis.

Introduction

The Metabolic Syndrome

Metabolic syndrome (MetSyn) is a term used to describe risk factors associated with the increased risk of chronic diseases including cardiovascular disease, type 2 diabetes (T2DM), and certain forms of cancer (*1-3*). MetSyn is an advancing epidemic that has become more prevalent in occurrence throughout the United States and other developed countries. Insulin resistance, dyslipidemia and obesity are important factors that define the metabolic syndrome. Age, genetics, hormonal change and lack of exercise are also considered to influence or exacerbate the syndrome (*1-3*). Specifically, insulin resistance, even independent of obesity, is a central underpinning of many chronic diseases and typically manifests several decades prior to disease onset (*4*). Within recent years many of the insulin receptor-signaling pathways have been uncovered, but there still remains an incomplete understanding of the underlying mechanisms contributing to the pathogenesis of impaired insulin action.

Skeletal muscle plays a central role in the development of T2DM, as it is a primary tissue involved in fatty acid metabolism and responsible for approximately 80% of insulin-stimulated glucose disposal (*5, 6*). There is considerable evidence suggesting that mitochondrial dysfunction and impairment of the oxidative capacity in skeletal muscle are key mechanisms for promoting insulin resistance (*6, 7*). Exercise and caloric restriction have been shown to

ameliorate features of the MetSyn in part by improving insulin sensitivity and overall metabolic function (*8, 9*). Improved metabolism and insulin action is thought to occur in response to enhanced mitochondrial functional capacity, number, and mtDNA integrity (*7, 10, 11*).

Mitochondrial dynamics

Mitochondria are highly dynamic double-membrane organelles that are responsible for energy production (*12*). The dynamic interplay of fission and fusion between mitochondria controls organelle integrity (i.e. oxidative capacity), morphology, size, and number (*13*). During mitochondrial biogenesis genetic mutations are likely to arise and proteins of functional significance are unequally distributed between daughter organelles. It is speculated that these potentially harmful occurrences result in a reduction of respiratory capacity and mtDNA levels (*11*). Mitochondrial fusion appears to provide a protective mechanism by allowing for mixing of mitochondrial contents and promoting a uniformed population of organelles. Fusion is also observed during certain cellular stresses; during nutrient starvation, mitochondria will fuse together as a prosurvival response to avoid degradation (mitophagy), and to increase ATP production (*14*).

Fusion and fission are also important means for mitochondria to ameliorate the effects of Reactive Oxygen Species (ROS). ROS are produced during normal respiration, primarily by complex I, and are increasingly produced during high demands of oxidative phosphorylation. ROS inflict oxidative damage on mitochondria and contribute to retrograde redox signaling (*15*). Damage

sustained by ROS subsequently increase further production of ROS, thus exacerbating organelle damage (*16*). Low levels of damaged mitochondria can undergo fusion complementation of content or undergo fission to eliminate 'beyond-repair' damaged contents (*17*).

Fission is also considered to be part of this quality control process. Much of the research on fission describes it as a critical process in providing growing and dividing cells with adequate numbers of mitochondria (*18*). Damaged mitochondria that have lost membrane potential, and have therefore become dysfunctional, undergo fission. The resulting triaged fragments may be targeted for degradation by the serine/threonine kinase PINK1 and the E3 ubiquitin ligase Parkin pathway (*19*). The theory behind fission in this instance is that mitochondria will be fragmented into smaller pieces to facilitate motility and engulfment by the autophagosome.

The size and shape of mitochondria are representative of their ongoing dynamics and the metabolic demands of the cell. When mitochondrial fusion rates are reduced, the mitochondrial population fragments into short tubules or small spheres because of ongoing mitochondrial fission. Reciprocally, increased mitochondrial fusion leads to elongated tubular morphology with improved oxidative capacity due to increased inner mitochondrial membrane (IMM) surface area and mixing of IMM content. These observations support the idea that mitochondrial morphology is dictated by a balance between fusion and fission (*13*).

Regulation of mitochondrial dynamics

Proteins located on mitochondrial inner and outer membranes mediate these opposing processes of fission and fusion. GTPase fusion proteins Mitofusin1 (Mfn1) and Mitofusin2 (Mfn2) are located on the mitochondrial outer membrane (OMM), and Optic Atrophy1 (Opa1) is located on the inner (IMM). Thus, Mfn1 and Mfn2 mediate the fusion of two mitochondrial OMMs, and Opa1 mediates the fusion of the IMMs (*13, 19*). Knockout of Mfn1, Mfn2, or Opa1 results in small fragmented mitochondria, indicating that these proteins are necessary for normal tubular morphology and maintenance of metabolic function.

Dynamin-related protein 1 (Drp1), also a GTPase, is one of the main mediators of fission. Drp1 is a cytosolic protein recruited by members of a class of receptors on the OMM to initiate mitochondrial fission. Drp1 is highly regulated by posttranslational covalent modifications including phosphorylation, sumoylation, ubiquitination, and S-nitrosylation (*20*). Phosphorylation sites Ser637 and Ser616 have been shown to be the primary regions of Drp1 activity modulation. These two phosphorylation sites act in opposing fashion: where phosphorylated Ser637 inhibits fission by restraining the translocation of Drp1 to the mitochondria (in other words, it promotes the retention of Drp1 in the cytosol), phosphorylated Ser616 promotes fission (*20, 21*). Current evidence suggests Drp1 can bind to four known OMM receptors: Mitochondrial fission 1 protein (Fis1), Mitochondrial fission factor (Mff), and Mitochondrial dynamics proteins 49 and 51 kDa (MiD 49 and 51, respectively).

Despite knowledge of these receptors, their exact mechanisms of Drp1 recruitment and receptor-specific binding to Drp1 remain unknown. Loson *et al*. recently assessed several mitochondrial outer membrane proteins for their ability to recruit Drp1 and initiate fission (*22*). It was shown in their experiments that Fis1 and Mff may act collectively or may function independently of one another to regulate mitochondrial fission. The MiDs 49 and 51 can mediate mitochondrial fission in the absence of Fis1 and Mff, but were proven non-essential as fission was fully stimulated in their absence (*21-23*). They also concluded that fission initiation may vary due to cell type, and cellular conditions may result in preferential binding. Their work identifies Fis1 and Mff as essential components of the mechanism of mitochondrial fission, and provides a basis for further study on the specific mechanisms of Drp1 recruitment.

Exercise influence on mitochondrial dynamics

It has been long established that exercise increases mitochondrial biogenesis and oxidative phosphorylation in association with increased expressions of regulatory factors such as peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α), nuclear respiratory factors 1 and 2 (NRF1 and 2), and TFAM (*24*). Exercise also alters the mitochondrial number, shape, and quality. Increases in mitochondrial biogenesis are thought by some to underlie the improvements in metabolism and insulin sensitivity seen in diabetic models [5, 6, 19, 22].

However, despite the recent boom of mitochondrial dynamics and metabolic research, much remains unknown about exercise-induced alterations in mitochondrial dynamics. Catoni *et al*. were the first to present findings linking exercise to changes in mitochondrial dynamic machinery, by specifically focusing on the responses and function of Mfn1/2. Soon after, another study suggested that gene expression of mitochondrial fusion and fission proteins in skeletal muscle can respond rapidly to increased metabolic demands during prolonged exercise (*25*). Rats performing acute single bouts of treadmill running of various durations showed reductions in *Mfn1/2* and increased *Fis1* mRNA expression and protein (*26*). Current findings also point to a correlation between increases in both ROS and mitochondrial respiratory function and changes in *Fis1* expression.

Following these works, it was proposed by Bori *et al*. and Ding *et al*. that increases in Fis1 would indeed result in increased exercise-induced mitochondrial fission. However, this work neglects the fact that Fis1 cannot self-initiate fission, and that a simple increase in mRNA expression or protein level is not definitive of mitochondrial fission. Therefore, I set out to attain more definitive evidence of the occurrence of mitochondrial fission, and uncover the mechanism of this process. I hypothesized that the recruitment of Drp1 by Fis1 (a protein-level interaction between Fis1 and Drp1) is the essential step for mitochondrial fission to take place.

Methods and Materials

Acute Exercise Study Animals and Time Course

Wild type C57Bl6/J female mice (n=24) were purchased from The Jackson Laboratory. Mice were allowed to acclimate to treadmill running (Columbus Instruments) using two bouts of exercise, each of 15-minute duration. Mice were fasted for 3 hours prior to experimentation. Sedentary control mice (Con, n=6) were placed on a non-running treadmill for 30 minutes. Two of the exercise-grouped mice (EXC) were subjected to treadmill running for 45 minutes (EXC45, n=6) and 90 minutes (EXC90, n=6). The third group of exercised mice was given a 3-hour recovery period following a 90 minute run (POST90, n=6). Treadmill speeds were gradually increased in the following chronological manner: time 0-1min at 5 m/min; 1-5min at 10 m/min; 5-10min at 15 m/min; and 10-45min (or 90min) at 18m/min. Post-experimentation, mice were euthanized with isoflurane gas. Blood plasma and tissue were collected and flash frozen for subsequent analysis.

All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Animal Subjects Committee of the University of California, Los Angeles.

Quantitative RT-PCR (qPCR)

Mouse tissues (~50mg each) were first homogenized using TRIzol reagent (Invitrogen), and RNA was isolated and further cleaned using RNeasy columns

(Qiagen) with DNase digestion. cDNA synthesis was performed using 3–6 μg of RNA with SuperScript II reverse transcriptase (Invitrogen). PCRs were prepared using iQ SYBR Green Super-mix (Bio-Rad). All PCRs were performed using a Bio-Rad MyiQ real time detection system. Quantification of a given gene, expressed as relative mRNA level compared with a control, was calculated after normalization to standard housekeeping gene Cyclophillin A (Ppia). Primer pairs were designed using Primer Express 2.0 software (Applied Biosystems). Primer sets were selected spanning at least one exon-exon junction when possible, and were checked for specificity using BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information). The specificity of the PCR amplification was confirmed by melting curve analysis, ensuring that a single product with its characteristic melting temperature was obtained. Primer sequences for the specific target genes analyzed are in Table 1.

Immunoblot Analysis

Skeletal muscle (quadriceps) tissues were first pulverized in liquid nitrogen and homogenized in RIPA lysis buffer (Milipore) containing freshly added protease (complete EDTA-Free, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2, Sigma). All lysates were clarified and centrifuged. Protein levels were determined using the Bradford assay (Bio-Rad). Lysates were heated at 95°C for 5min in Laemmli buffer and resolved by SDS-PAGE. Samples were transferred to PVDF membranes and subsequently probed with the following antibodies for protein and phospho-protein detection: Fis1 and

Mfn2 (Abcam); Opa1 (BD biosciences); p-Drp1^{Ser637}, p-Drp1^{Ser616}, and Drp1 (Cell Signaling Technologies); Mff (gift from Alexander van der Bliek, UCLA); and GAPDH (Ambion). Whole tissue and cytosolic fractioned proteins were adjusted for background artefact and normalized to housekeeping GAPDH. Isolated mitochondrial fractioned proteins were also adjusted for background artefact and normalized to mitochondrial loading control Porin (MitoSciences). Densitometric analysis was performed using Bio-Rad Chemidoc Quantity One imaging software (Version 4.6).

Mitochondrial Isolation

Mitochondria were isolated using the Mitochondrial Isolation Kit for Tissue (Thermo/Pierce) according to the Manufacturer with the following modifications:

Whole quadriceps weighing between 128-131mg were finely minced and incubated for 3 minutes in 0.8mg/ml of trypsin/PBS. Samples were then homogenized with a dounce-homogenizer using ~4 strokes. Mitochondria were pelleted at 12,000 x *g* and resuspended in 200μl of RIPA lysis buffer. Downstream processing and analysis of proteins were performed as mentioned in the *Immunoblot Analysis* section.

Table 1. qRT-PCR primer sequences.

Gene Symbol	Forward (5'-3')	Reverse (5'-3')
Esr1	GCTACTGTGCCGTGTGCAA	TGTCAATGGTGCATTGGTTTG
Pgc1a	TGAGGACCGCTAGCAAGTTT	TGAAGTGGTGTAGCGACCAA
Ppia	AGCCAAATCCTTTCTCTCCAG	CACCGTGTTCTTCGACATCA
Mfn1	GCTTCCGACGGACTTACAAC	TGAATAACCGTTGGGATGCT
Mfn2	ATTGATCACGGTGCTCTTCC	GTCCTGGACGTCAAAGGGTA
Mff	GCAGTTGGCAGGCTAAAAAG	TCAGGTAGCATATGGGGAGG
Mef2c	GCCGGACAAACTCAGACATTG	GGGTTTCCCAGTGTGCTGAC
Rcan1	TGTGGCAAACGATGATGTCT	GCAGATAAGGGGTTGCTGAA
Sqstm1	TCTGGGGTAGTGGGTGTCAG	AGAATGTGGGGGAGAGTGTG
IL6	AGTCCGGAGAGGAGACTTCA	TTGCCATTGCACAACTCTTT
CnA	GCGATTGATCCCAAGTTGTC	TGCCCTCCTTCATGAGATGT
CS	AAGGACGAGGCAGGATGAG	TGCAGCTGTAGCTCTCTCCC
Fhl2	AGGAGTGTGGAACACCCATC	AGCTGTTCCTCCTTGGCAG
Fis1	AGGAGCTGGACCGCCTGATTG	AGGATTTGGACTTGGAGACAG
Gpx3	GATGGTGAGGGCTCCATACT	CATCCTGCCTTCTGTCCCT
Hsp90aa1	TAACTCCGCCTTTGTGGAA	TCTTGCCCTCAAATTCCTTC
HspA1a	CAAGAAGAAGGTGCTGGACA	GGTACAGCCCACTGATGATG
HspA1b	CAAGAATGCGCTCGAGTCCTA	GGAGATGACCTCCTGGCACTT

Results

RNA Expression: Markers of Exercise and Stress

Mouse quadriceps muscles were assessed for changes in gene expression. Markers of exercise and cellular stress were determined to ascertain whether animals underwent a sufficient level of exercise. Calcineurin (CnA) RNA expression increased by 32% ($p<0.05$), and Regulator of Calcineurin1 (*Rcan1*) increased by ~6 fold in exercised 90min animals (EXC90) and 3hr post-90min exercised animals (POST90) ($p<0.005$); this indicates that Calcineurin had been activated, and therefore a sufficient level of exercise had been reached. The expression of stress response molecular chaperone Heat Shock 70 kDa protein 1A (*HspA1a*), Heat shock 70 kDa protein 1B (*HspA1b*), and Heat Shock Protein 90A (*Hsp90*) all increased expression by: ~250 fold ($p<0.005$) in EXC90 and ~80 fold ($p<0.05$) in POST90; ~110 fold ($p<0.005$) in EXC90 and ~30 fold ($p<0.05$) in POST90; and ~1.5 fold ($p<0.05$) in EXC45 and ~3 fold ($p<0.005$) in EXC90, respectively (Supplementary Fig. 1 a-i). Expression of myogenic response genes *MyoD1* and *MyoG* were significantly elevated ~1.8 fold ($p<0.05$) in EXC90 animals, and reduced by ~45% ($p<0.05$) in POST90 animals (Supplementary Fig. 3 b and c).

RNA Expression: Markers of Mitochondrial Biogenesis and Dynamic Proteins

Mitochondrial biogenesis marker peroxisome proliferator activated receptor γ co-activator 1 α (*PGC1*α) showed a ~6 fold increase ($p<0.005$) for EXC90 and maintained the ~6 fold increase ($p<0.05$) 3h post exercise in

POST90. Mitochondrial fission receptor *Fis1* increased expression by ~2 fold ($p<0.005$) in both EXC90 and POST90 animals. Additionally, *Mff* expression was reduced by ~0.8 fold in POST90. Contrary to previous reports [18], I observed no significant changes in fusion protein genes *Mfn1* and *Mfn2* for any of the groups (Fig. 1 a-f).

Immunoblotting: Quadriceps muscle

Western analysis performed on quadriceps muscle showed a remarkable increase in phosphorylation of Dynamin-related protein 1 (Drp1) at Ser616 with longer exercise duration, whereas phosphorylation of Drp1 at Ser637 decreased over time (Fig. 2 a). A decrease in phosphorylation at site Ser637 was anticipated as exercise induces the activation of Calcineurin, a phosphatase known to target Drp1 at Ser637 (*27*).

Exercise-induced metabolic signaling was assessed by immunoblotting for phosphorylation of AMPKα at Thr172. Findings showed a gradual increase of phosphorylation throughout the exercise duration (Supplementary Fig. 2), as previously reported (*28*).

Immunoblotting: Isolated Mitochondria

Mitochondria from quadriceps muscles were isolated to gain understanding of the localization of fission signaling molecules during acute exercise. Immunoblots of phospho-Drp1 showed internal consistency with my observations for whole tissue lysates. Mitochondrial fractions showed an

increase of phospho-Drp1^{Ser616} and a decrease of phospho-Drp1^{Ser637} with exercise. Densitometric calculations were not performed because of limited sample numbers.

Mitochondrial Drp1 receptor proteins promoting fission, Mff and Fis1, were elevated with exercise progression (Fig. 4 a, c, & d). Mff increased ~1.5 fold ($p<0.05$) in EXC90 and POST90 animals (Fig. 4 c). Fis1 protein levels were elevated only in the muscle of the POST90 group (Fig. 4 d), findings in agreement with a recent report (*26*). Mitochondrial fusion promoting protein Opa1 was also elevated with exercise progression, with a 1.5 fold ($p<0.05$) increase in POST90 animals (Fig. 4 a & b).

Discussion

In this study I sought to characterize changes in expression and signaling of proteins involved in mitochondrial dynamics in response to acute exercise. Acute single bout exercise led to increased *Fis1* gene expression and protein levels. These observations are in agreement with findings in rat skeletal muscle following acute treadmill running (*26*). However I did not detect a notable decrease for fusion proteins Mfn1 and Mfn2 as previously described. Going beyond what had been studied by previous researchers, I tested to see if another mitochondrial outer membrane fission protein, Mff, was influenced by exercise. Mff has recently gained credit for playing an integral role in mitochondrial fission, and possibly more so then Fis1 (*22*). Indeed, my study showed that Mff protein levels on the mitochondria were increased with prolonged exercise.

A novel and interesting finding was the increase of Drp1^{Ser616} phosphorylation in the 90 minute exercise group (EXC90). Drp1^{Ser616} phosphorylation indicates that Drp1 is being recruited by proteins of the outer mitochondrial membrane, and therefore suggests that fission is occurring. However, this research could not identify exactly which OMM proteins were responsible for the recruitment of Drp1. One technique that may provide this important data on both the occurrence of mitochondrial fission and the involvement of particular proteins is co-immunoprecipitation (CoIP). Other researchers working in the same lab were unsuccessful in utilizing this technique. However, I hypothesize that a gentler washing of the mitochondrial membrane proteins, utilizing a different detergent, will allow me to isolate membrane

proteins in a more native conformation. Then, the CoIP technique may be more readily applied to these more intact proteins, revealing any affinity or preference of these proteins for known fission binding proteins. In any case, the most definitive means of concluding that fission is taking place is electron microscopy (EM) images. Unfortunately, neither of these techniques could be implemented in this study due to both time and financial constraints.

The dynamics between mitochondrial fusion and fission are an ongoing cycle where biogenesis and mitophagy collectively perform organelle triage, contribute to the quantity and quality of the mitochondria. The data from this study suggest that exercise increases the overall occurrence of cycling dynamics. This would explain why we observe increases in both fission and fusion protein expression. At 90 minutes of exercise, it appears that a threshold is met where changes in dynamic protein expression levels occur.

It is possible that near the 90 minute mark, mitochondria have been sufficiently stressed beyond repair, and thus undergo fission to remove damaged segments for subsequent mitophagy. Past experiments have highlighted the importance of fission-mediated mitophagy. Relatedly, a recent publication by Levine laboratory showed that autophagy is activated by 80 minutes of exercise (*28, 29*). Although the He *et al*. experiment did not investigate mitophagy during exercise per se, it would be interesting to compare the relative timing of fission and autophagy observed by He *et al*. and my experiments. However, it seems unreasonable to target mitochondria for autophagy in times of high metabolic demand.

Another explanation for increased fission during exercise is the remodeling of mitochondria in size and morphology to promote enhanced mobility. Recent studies of neurological diseases have suggested that mitochondrial fission is vital for generating mitochondria small enough to be transported through the narrow axons of neurons (*29, 30*). It is possible that a similar process may account for the high levels of fission in skeletal muscle due to acute exercise. Skeletal muscle is dense in substructure; it is possible that certain regions within the muscle have different metabolic demands and therefore may require the recruitment of additional mitochondria to satisfy the ATP requirements of the working cell. Taking into account the fact that fission is highly up-regulated during cellular development (myogenesis), it is plausible that small fragmented mitochondria possess an agility necessary to be taxied along the cytoskeleton (by Miro/Milton (*30-32*)) to particular regions of the muscle that are in metabolic need.

A decline in Opa1 protein could entail the targeting of fragmented mitochondria for mitophagy (*17, 33*). Of note is the interesting observation in my study of the sequential increase in the mitochondrial Opa1 protein levels with exercise. This observation may support the above theory that fission may indeed be favored for mitochondrial locomotive purposes. To add to this theory, a recent study assessed a group of exercised mice and found that, in mouse skeletal muscle, mitochondrial density was increased, as was the number of interactions between mitochondria (*34*). Remarkably, the authors report no significant changes in morphology, suggesting that mitochondrial may move about the

skeletal muscle in a ‘kiss and run’ pattern as mentioned in a study by Shirihai *et al*. (*17*).

As noted above, future studies of multi-plane sectioning of exercised skeletal muscle via electron microscopy would be required to glean a full perspective of changes in mitochondrial morphology. From a molecular-mechanistic point of view, it would be helpful to immunoprecipitate Drp1 and observe any binding with phosphorylation at sites Ser616 and Ser637 to determine the engagement of OMM receptors. Additionally, florescent labeling of mitochondria may also be helpful in investigating the density and locomotive theory of fragmented mitochondria.

The past decade has presented a surge of interesting findings in the field of mitochondrial dynamics. Although most of the research’s attention has been directed at the nervous system, we are starting to understand the significant roles mitochondrial dynamics may play in the development of other maladies such as diabetes and cardiovascular diseases (*35*). The discovery and understanding of these mitochondrial changes could prove instrumental in the development of therapeutic strategies to combat these chronic diseases that afflict our society.

References

1. R. A. DeFronzo, R. C. Bonadonna, E. Ferrannini, Pathogenesis of NIDDM. A balanced overview. *Diabetes Care* **15**, 318-368 (1992); published online EpubMar.

2. K. G. M. M. Alberti, R. H. Eckel, S. M. Grundy, P. Z. Zimmet, J. I. Cleeman, K. A. Donato, J. C. Fruchart, W. P. T. James, C. M. Loria, S. C. Smith, Harmonizing the Metabolic Syndrome A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* **120**, 1640-1645 (2009); published online EpubOct 20 (Doi 10.1161/Circulationaha.109.192644).

3. J. L. Rosenzweig, E. Ferrannini, S. M. Grundy, S. M. Haffner, R. J. Heine, E. S. Horton, R. Kawamori, S. Endocrine, Primary prevention of cardiovascular disease and type 2 diabetes in patients at metabolic risk: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* **93**, 3671-3689 (2008); published online EpubOct (10.1210/jc.2008-0222).

4. A. M. Johnson, J. M. Olefsky, The origins and drivers of insulin resistance. *Cell* **152**, 673-684 (2013); published online EpubFeb 14 (10.1016/j.cell.2013.01.041).

5. G. I. Shulman, D. L. Rothman, T. Jue, P. Stein, R. A. DeFronzo, R. G. Shulman, Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy. *The New England journal of medicine* **322**, 223-228 (1990); published online EpubJan 25 (10.1056/NEJM199001253220403).

6. J. Hoeks, P. Schrauwen, Muscle mitochondria and insulin resistance: a human perspective. *Trends in endocrinology and metabolism: TEM* **23**, 444-450 (2012); published online EpubSep (10.1016/j.tem.2012.05.007).

7. E. Phielix, R. Meex, E. Moonen-Kornips, M. K. Hesselink, P. Schrauwen, Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in

control individuals. *Diabetologia* **53**, 1714-1721 (2010); published online EpubAug (10.1007/s00125-010-1764-2).

8. E. Teixeira-Lemos, S. Nunes, F. Teixeira, F. Reis, Regular physical exercise training assists in preventing type 2 diabetes development: focus on its antioxidant and anti-inflammatory properties. *Cardiovasc Diabetol* **10**, 12 (2011)10.1186/1475-2840-10-12).

9. D. E. Larson-Meyer, L. K. Heilbronn, L. M. Redman, B. R. Newcomer, M. I. Frisard, S. Anton, S. R. Smith, A. Alfonso, E. Ravussin, Effect of calorie restriction with or without exercise on insulin sensitivity, beta-cell function, fat cell size, and ectopic lipid in overweight subjects. *Diabetes Care* **29**, 1337-1344 (2006); published online EpubJun (10.2337/dc05-2565).

10. B. B. Lowell, G. I. Shulman, Mitochondrial dysfunction and type 2 diabetes. *Science* **307**, 384-387 (2005); published online EpubJan 21 (10.1126/science.1104343).

11. H. Chen, M. Vermulst, Y. E. Wang, A. Chomyn, T. A. Prolla, J. M. McCaffery, D. C. Chan, Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell* **141**, 280-289 (2010); published online EpubApr 16 (10.1016/j.cell.2010.02.026).

12. I. E. Scheffler, *Mitochondria*. (Wiley-Liss, 2008).

13. D. C. Chan, Fusion and fission: interlinked processes critical for mitochondrial health. *Annual review of genetics* **46**, 265-287 (2012)10.1146/annurev-genet-110410-132529).

14. A. S. Rambold, B. Kostelecky, N. Elia, J. Lippincott-Schwartz, Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 10190-10195 (2011); published online EpubJun 21 (Doi 10.1073/Pnas.1107402108).

15. M. P. Murphy, How mitochondria produce reactive oxygen species. *Biochem J* **417**, 1-13 (2009); published online EpubJan 1 (10.1042/BJ20081386).

16. R. S. Balaban, S. Nemoto, T. Finkel, Mitochondria, oxidants, and aging. *Cell* **120**, 483-495 (2005); published online EpubFeb 25 (10.1016/j.cell.2005.02.001).

17. G. Twig, A. Elorza, A. J. Molina, H. Mohamed, J. D. Wikstrom, G. Walzer, L. Stiles, S. E. Haigh, S. Katz, G. Las, J. Alroy, M. Wu, B. F. Py, J. Yuan, J. T. Deeney, B. E. Corkey, O. S. Shirihai, Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO J* **27**, 433-446 (2008); published online EpubJan 23 (10.1038/sj.emboj.7601963).

18. N. Taguchi, N. Ishihara, A. Jofuku, T. Oka, K. Mihara, Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. *The Journal of biological chemistry* **282**, 11521-11529 (2007); published online EpubApr 13 (10.1074/jbc.M607279200).

19. R. J. Youle, A. M. van der Bliek, Mitochondrial fission, fusion, and stress. *Science* **337**, 1062-1065 (2012); published online EpubAug 31 (10.1126/science.1219855).

20. C. R. Chang, C. Blackstone, Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. *Annals of the New York Academy of Sciences* **1201**, 34-39 (2010); published online EpubJul (10.1111/j.1749-6632.2010.05629.x).

21. C. S. Palmer, L. D. Osellame, D. Laine, O. S. Koutsopoulos, A. E. Frazier, M. T. Ryan, MiD49 and MiD51, new components of the mitochondrial fission machinery. *EMBO reports* **12**, 565-573 (2011); published online EpubJun (10.1038/embor.2011.54).

22. O. C. Loson, Z. Song, H. Chen, D. C. Chan, Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. *Mol Biol Cell* **24**, 659-667 (2013); published online EpubMar (10.1091/mbc.E12-10-0721).

23. C. S. Palmer, K. D. Elgass, R. G. Parton, L. D. Osellame, D. Stojanovski, M. T. Ryan, Adaptor proteins MiD49 and MiD51 can act independently of Mff and Fis1 in Drp1 recruitment and are specific for mitochondrial fission. *The Journal of biological chemistry* **288**, 27584-27593 (2013); published online EpubSep 20 (10.1074/jbc.M113.479873).

24. Z. Bori, Z. Zhao, E. Koltai, I. G. Fatouros, A. Z. Jamurtas, Douroudos, II, G. Terzis, A. Chatzinikolaou, A. Sovatzidis, D. Draganidis, I. Boldogh, Z. Radak, The effects of aging, physical training, and a single bout of exercise on mitochondrial protein expression in human skeletal muscle. *Experimental gerontology* **47**, 417-424 (2012); published online EpubJun (10.1016/j.exger.2012.03.004).

25. R. Cartoni, B. Leger, M. B. Hock, M. Praz, A. Crettenand, S. Pich, J. L. Ziltener, F. Luthi, O. Deriaz, A. Zorzano, C. Gobelet, A. Kralli, A. P. Russell, Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. *The Journal of physiology* **567**, 349-358 (2005); published online EpubAug 15 (10.1113/jphysiol.2005.092031).

26. H. Ding, N. Jiang, H. Liu, X. Liu, D. Liu, F. Zhao, L. Wen, S. Liu, L. L. Ji, Y. Zhang, Response of mitochondrial fusion and fission protein gene expression to exercise in rat skeletal muscle. *Biochimica et biophysica acta* **1800**, 250-256 (2010); published online EpubMar (10.1016/j.bbagen.2009.08.007).

27. G. M. Cereghetti, A. Stangherlin, O. Martins de Brito, C. R. Chang, C. Blackstone, P. Bernardi, L. Scorrano, Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 15803-15808 (2008); published online EpubOct 14 (10.1073/pnas.0808249105).

28. C. He, M. C. Bassik, V. Moresi, K. Sun, Y. Wei, Z. Zou, Z. An, J. Loh, J. Fisher, Q. Sun, S. Korsmeyer, M. Packer, H. I. May, J. A. Hill, H. W. Virgin, C. Gilpin, G. Xiao, R. Bassel-Duby, P. E. Scherer, B. Levine, Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature* **481**, 511-515 (2012); published online EpubJan 26 (10.1038/nature10758).

29. A. M. Choi, S. W. Ryter, B. Levine, Autophagy in human health and disease. *The New England journal of medicine* **368**, 651-662 (2013); published online EpubFeb 14 (10.1056/NEJMra1205406).

30. M. van Spronsen, M. Mikhaylova, J. Lipka, M. A. Schlager, D. J. van den Heuvel, M. Kuijpers, P. S. Wulf, N. Keijzer, J. Demmers, L. C. Kapitein, D. Jaarsma, H. C. Gerritsen, A. Akhmanova, C. C. Hoogenraad, TRAK/Milton

motor-adaptor proteins steer mitochondrial trafficking to axons and dendrites. *Neuron* **77**, 485-502 (2013); published online EpubFeb 6 (10.1016/j.neuron.2012.11.027).

31. A. Weihofen, K. J. Thomas, B. L. Ostaszewski, M. R. Cookson, D. J. Selkoe, Pink1 forms a multiprotein complex with Miro and Milton, linking Pink1 function to mitochondrial trafficking. *Biochemistry* **48**, 2045-2052 (2009); published online EpubMar 10 (10.1021/bi8019178).

32. X. Wang, D. Winter, G. Ashrafi, J. Schlehe, Y. L. Wong, D. Selkoe, S. Rice, J. Steen, M. J. LaVoie, T. L. Schwarz, PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell* **147**, 893-906 (2011); published online EpubNov 11 (10.1016/j.cell.2011.10.018).

33. I. Kim, J. J. Lemasters, Mitochondrial degradation by autophagy (mitophagy) in GFP-LC3 transgenic hepatocytes during nutrient deprivation. *Am J Physiol Cell Physiol* **300**, C308-317 (2011); published online EpubFeb (10.1152/ajpcell.00056.2010).

34. M. Picard, B. J. Gentil, M. J. McManus, K. White, K. St Louis, S. E. Gartside, D. C. Wallace, D. M. Turnbull, Acute exercise remodels mitochondrial membrane interactions in mouse skeletal muscle. *J Appl Physiol*, (2013); published online EpubAug 22 (10.1152/japplphysiol.00819.2013).

35. S. B. Ong, A. R. Hall, D. J. Hausenloy, Mitochondrial dynamics in cardiovascular health and disease. *Antioxidants & redox signaling* **19**, 400-414 (2013); published online EpubAug 1 (10.1089/ars.2012.4777).

Figures

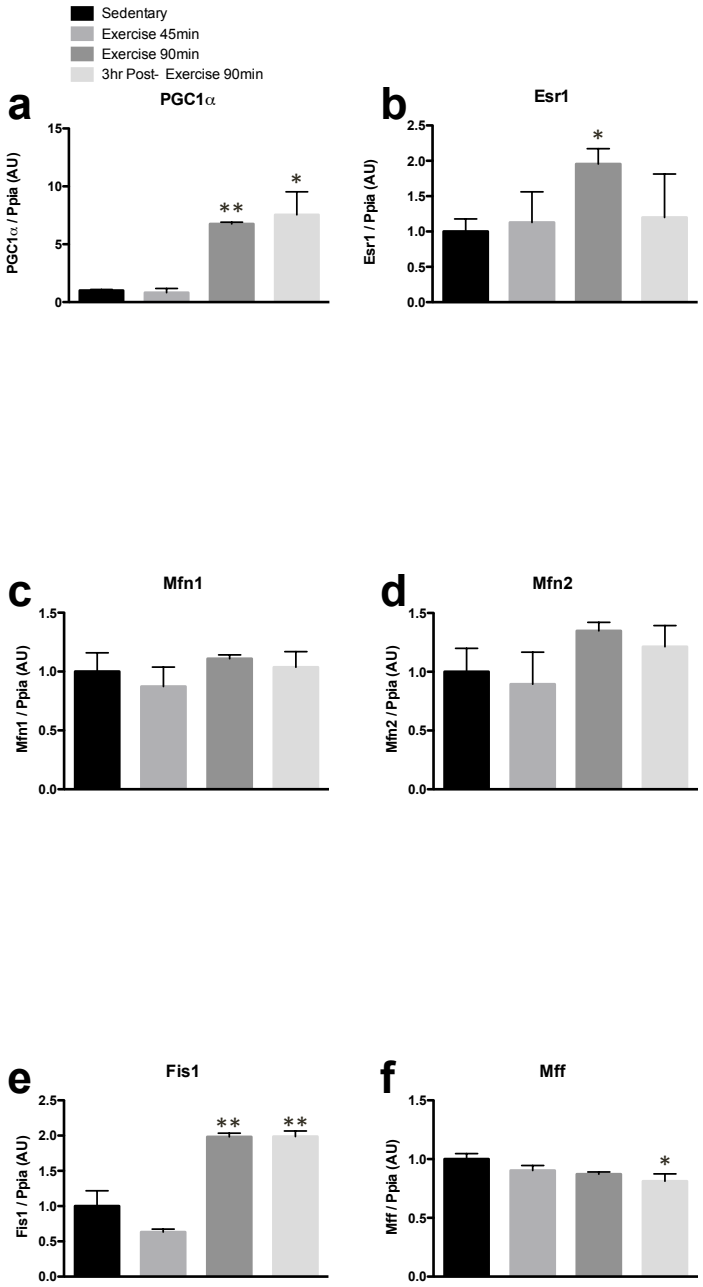


Figure 1- Modifiers of Mitochondrial Biogenesis and Dynamics: Gene Expression. Quantification of a given gene, expressed as relative mRNA level compared with Sedentary control, was calculated after normalization to standard housekeeping gene Cyclophillin A (Ppia) with * $p<0.05$, ** $p<0.005$. n= 6 each group.

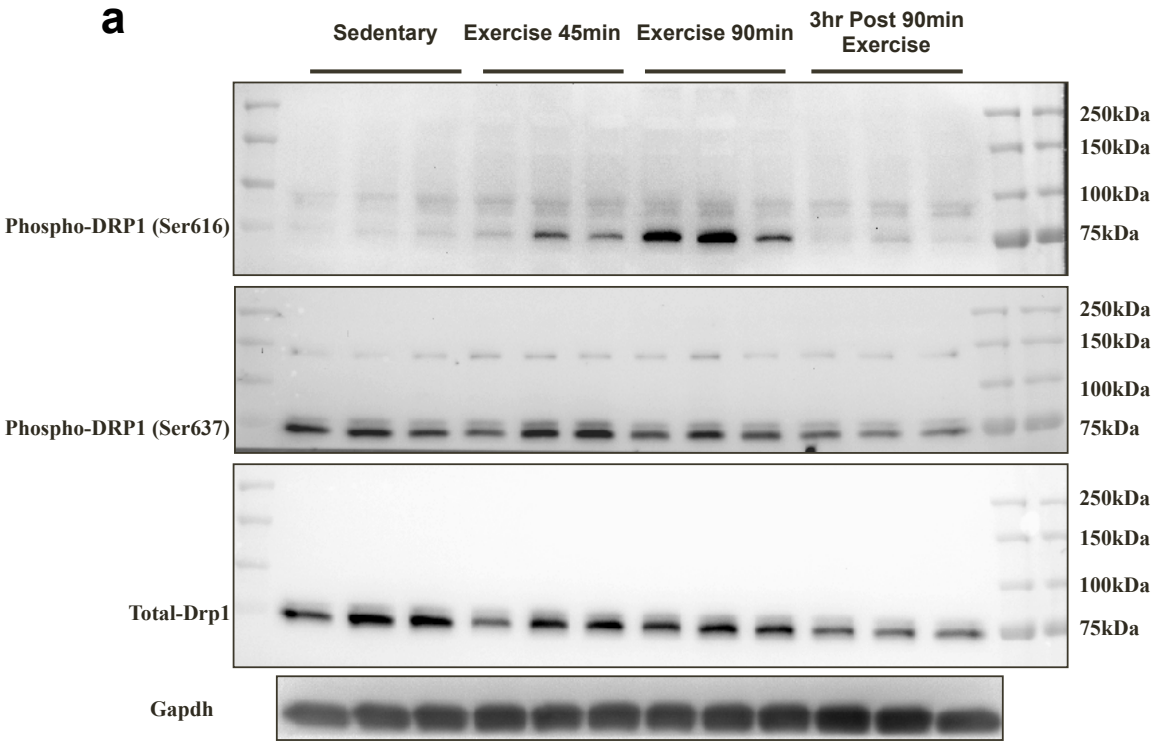


Figure 2- Exercise Induced Changes in Dynamin-related protein 1 (Drp1) Phosphorylation: Western Blot (a) and Densitometry (b-c). Quantification of phosphorylated Drp1 is normalized to the total protein and housekeeping loading control Gapdh with * $p<0.05$. n= 6 each group (showing n=3).

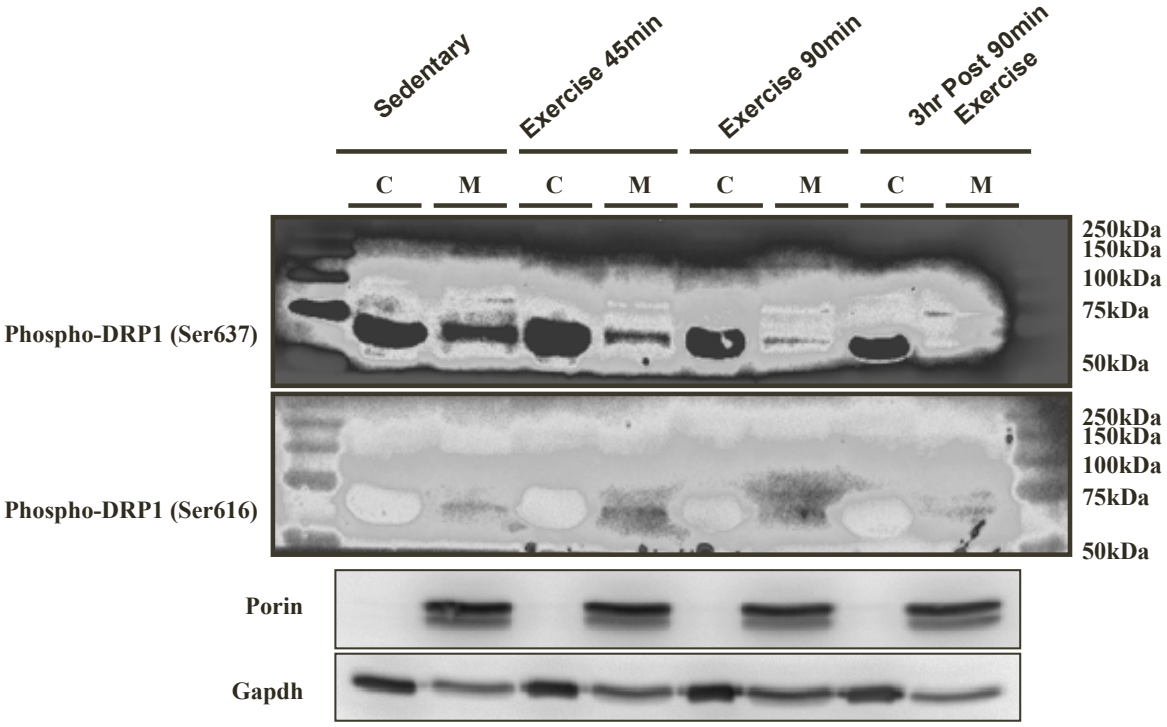


Figure 3- Exercise Induced Changes in Dynamin-related protein 1 (Drp1) Phosphorylation from Isolated Mitochondria (M) and Cytosolic Fractions (C): Western Blot. No densitometry/ quantification of protein available due to an incomparable total Drp1 protein image. n= 3 each group (showing n=1).

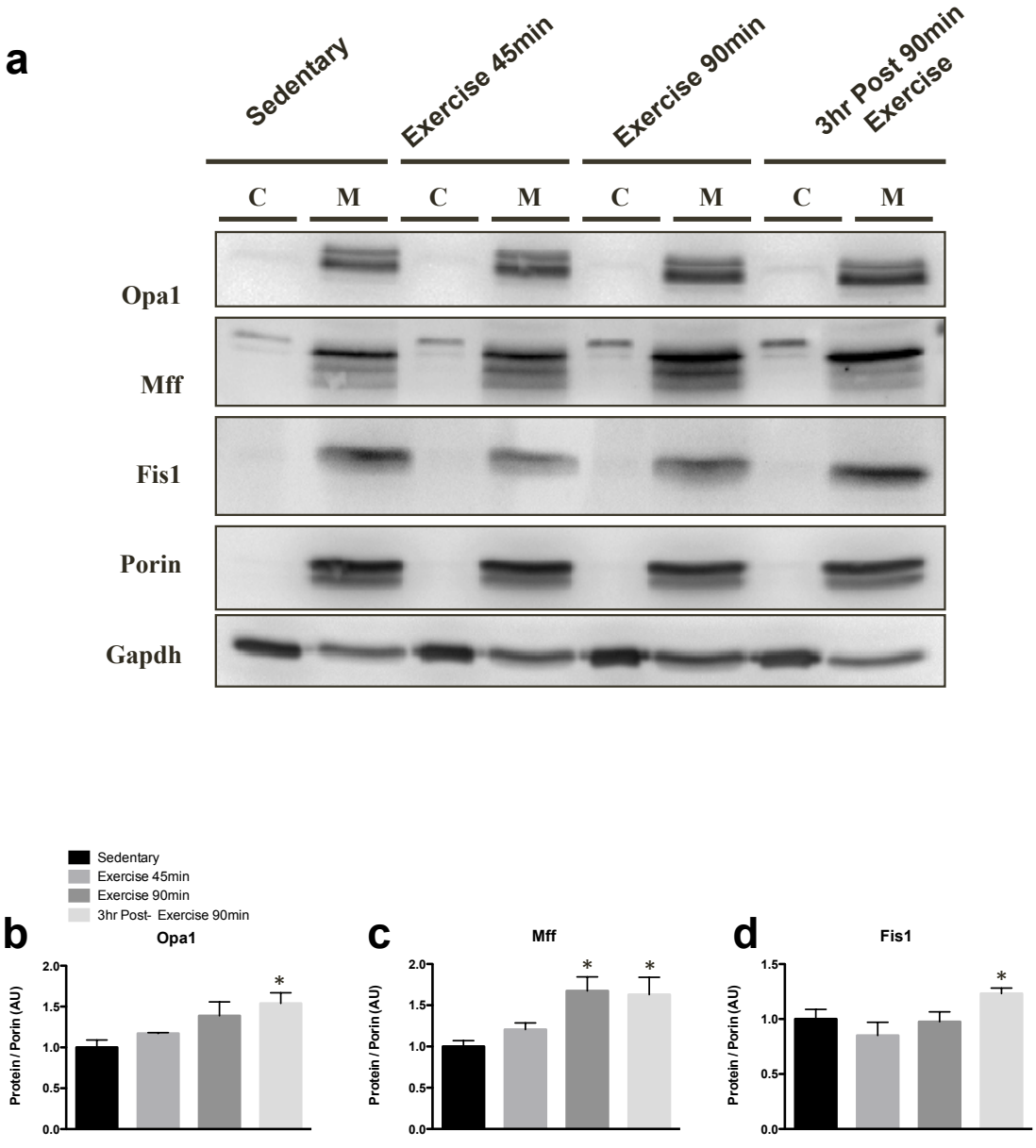
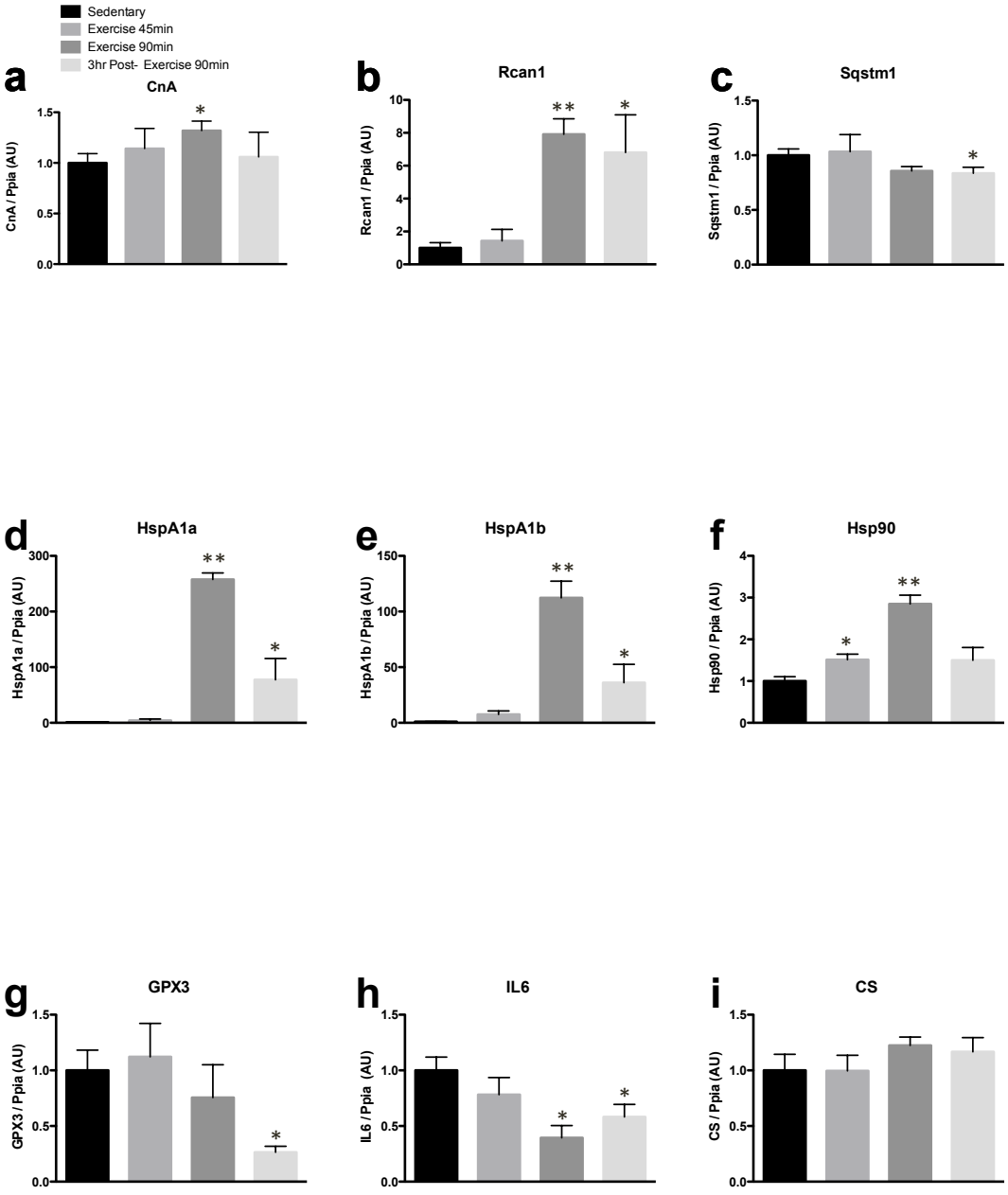
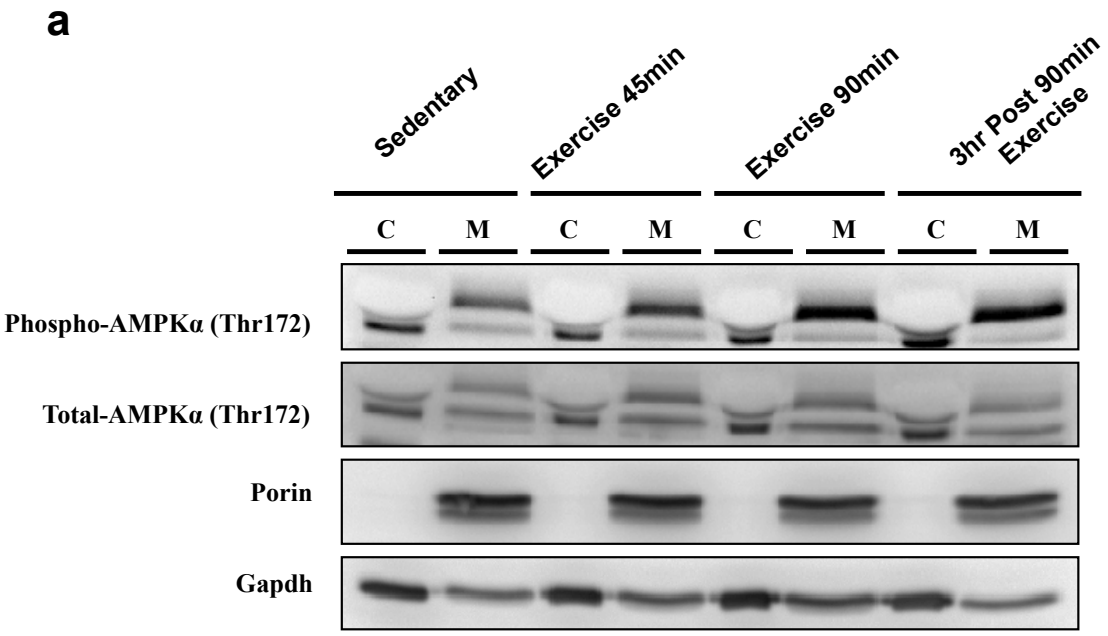


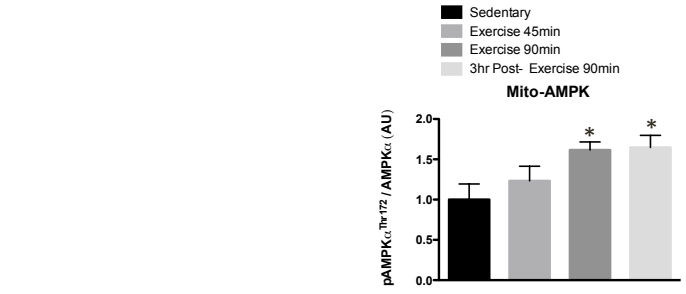
Figure 4- Exercise Induced Changes in Other Fission/Fusion Proteins from Isolated Mitochondria: Western Blot (a) and Densitometry (b-d). Densitometry/quantification of protein is normalized to mitochondrial loading control Porin with * $p<0.05$. n= 3 each group (showing n=1).

Supplementary Figures

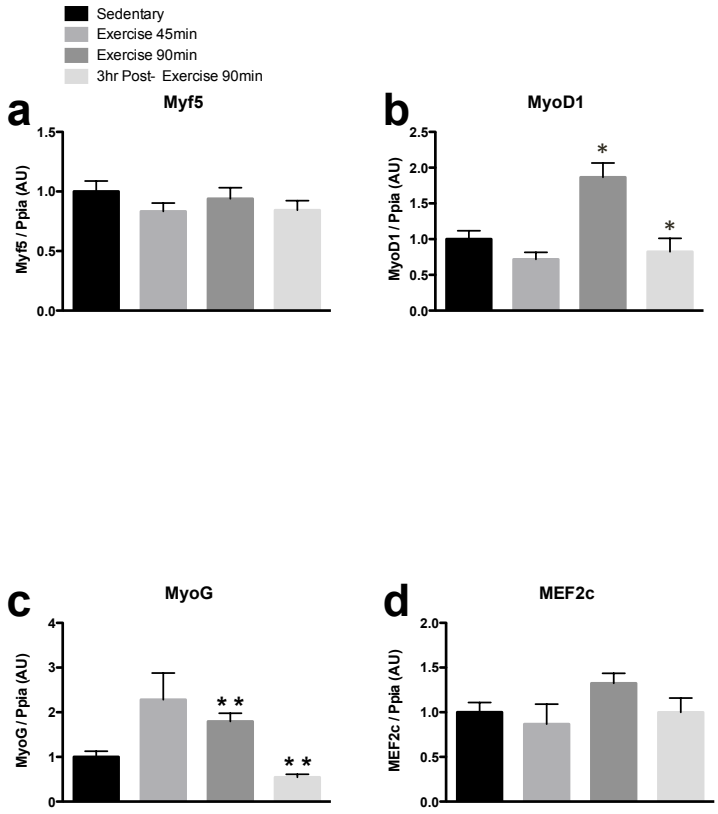


Supplementary Figure 1- Markers of Stress and Exercise: Gene Expression. Quantification of a given gene, expressed as relative mRNA level compared with Sedentary control, was calculated after normalization to standard housekeeping gene Cyclophillin A (Ppia) with * $p<0.05$, ** $p<0.005$. n= 6 each group.





Supplementary Figure 2- Exercise Induced changes in AMPK from Isolated Mitochondria (M) and cytosolic fractions (C): Western Blot (a) and Densitometry (b). Densitometry/quantification of phosphorylated protein is normalized to total protein and mitochondrial loading control Porin with * $p<0.05$. n= 3 each group (showing n=1).



Supplementary Figure 3- Markers of Myogenesis: Gene Expression**.** Quantification of a given gene, expressed as relative mRNA level compared with Sedentary control, was calculated after normalization to standard housekeeping gene Cyclophillin A (Ppia) with * $p<0.05$, ** $p<0.005$. n= 6 each group.