UC Berkeley
UC Berkeley Electronic Theses and Dissertations

Title

Mild Heat Stress Induces Mitochondrial Biogenesis Associated with Activation of the AMPK-SIRT1-PGC-1alpha Pathway in C2C12 Myotubes

Permalink

https://escholarship.org/uc/item/1s48q979

Author

Liu, Chien-Ting

Publication Date

2011

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

Mild Heat Stress Induces Mitochondrial Biogenesis Associated with Activation of the AMPK-SIRT1-PGC-1α Pathway in C2C12 Myotubes

By

Chien-Ting Liu

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor George A. Brooks, Chair

Professor Steven L. Lehman

Professor Sharon E. Fleming

Fall 2011

ABSTRACT

Mild Heat Stress Induces Mitochondrial Biogenesis Associated with Activation of the AMPK-SIRT1-PGC-1α Pathway in C2C12 Myotubes

By

Chien-Ting Liu

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor George A. Brooks, Chair

During endurance exercise, most (≈ 75%) of the energy derived from the oxidation of metabolic fuels and ATP hydrolysis of muscle contraction is liberated as heat, the accumulation of which leads to an increase in body temperature. For example, the temperature of exercising muscles can rise to 40°C. Although severe heat injury can be deleterious, several beneficial effects of mild heat stress (HS), such as the improvement of insulin sensitivity in patients with type 2 diabetes have been reported. However, among all cellular events induced by mild HS from physical activities, the direct effects and mechanisms of mild HS on mitochondrial biogenesis in skeletal muscle are least characterized. AMP-activated protein kinase (AMPK) and Sirtuin 1 (SIRT1) are key energy-sensing molecules regulating mitochondrial biogenesis. In C2C12 myotubes, we found that one-hr mild HS at 40°C increased both AMPK activity and SIRT1 expression, as well as increased the expression of several mitochondrial biogenesis regulatory genes including peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and transcription factors involved in mitochondrial biogenesis. In particular, PGC-1α expression was found to be transcriptionally regulated by mild HS. Additionally, after repeated mild HS for 5 days, protein levels of PGC-1α and several mitochondrial oxidative phosphorylation subunits also increased. Repeated mild HS also significantly increased mitochondrial DNA copy number. In conclusion, these data suggest that mild HS is sufficient to induce mitochondrial biogenesis associated with activation of the AMPK-SIRT1-PGC-1α pathway in C2C12 myotubes. Therefore, it is possible that muscle heat production during exercise plays a role in mitochondrial biogenesis.

DEDICATION

This dissertation is dedicated to my wonderful family (Jing-Yi and Wen-Ning) who give me the inspiration, love and encouragement to live my dreams, and to the rest of my family in Taiwan who also provided unconditional love and support.

TABLE OF CONTENTS

ABSTRACT……1

DEDICATION……i

TABLE OF CONTENTS……ii

LIST OF TABLES AND FIGURES……iii

ACKNOWLEDGEMENTS……v

LIST OF ABBREVIATIONS……vi

CHAPTER 1 INTRODUCTION……1

A. Review of the literature……2

B. Hypothesis……8

CHAPTER 2 MATERIALS AND METHODS……15

CHAPTER 3 RESULTS……20

A. Mild heat stress increases AMPK activity and SIRT1 expression……21

B. Mild heat stress increases PGC-1α transcription……26

C. Mild heat stress activates mitochondrial biogenesis program……31

D. Mild heat stress induces gene expression for mitochondrial components……37

E. Mild heat stress induces cytoprotective heat shock response……42

F. Repeated mild heat stress increases the expression of PGC-1α and oxidative phosphorylation subunit proteins as well as mitochondrial DNA copy number……47

CHAPTER 4 DISCUSSION……52

CHAPTER 5 CONCLUSION AND FUTURE DIRECTIONS……56

A. Conclusion……57

B. Future Directions……58

REFERENCES……61

LIST OF TABLES AND FIGURES

Table 1: Main effects of physical and metabolic signals associated with endurance exercise……………………………………………………………………….....9

Table 2: Primer sequences used for real-time quantitative PCR………………………...…19

Figure 1: Metabolic changes induced by AMPK in a variety of tissues.……………………..10

Figure 2: SIRT1 coordinates metabolic responses to nutritional availability in various tissue………………………………………………………………………….11

Figure 3: PGC1α regulatory cascade…………………………………………………………12

Figure 4: Schematic representation of mitochondrial biogenesis by the AMPK-SIRT1-PGC-1α pathway….……………………………………………...…………13

Figure 5A: Mild HS up-regulates AMPK activity……………………………………………22

Figure 5A: Mild HS up-regulates SIRT1 expression……………………………………….23

Figure 5C: Mild HS increases Sirt1 mRNA expression………………………………………24

Figure 5D: Mild HS up-regulates Nampt expression…………………………………………25

Figure 6A: Mild HS increases *PGC-1α* mRNA expression…………………………………27

Figure 6B: *PGC-1β* mRNA expression after Mild HS………………………………………28

Figure 6C: *PRC* mRNA expression after Mild HS…………………………………………...29

Figure 6D: Mild HS increases *PGC-1α* transcription………………………………………...30

Figure 7A: Mild HS activates mitochondrial biogenesis program (*Nrf1*)…….……………...32

Figure 7B: Mild HS activates mitochondrial biogenesis program (*Nrf2α*)………………..…..33

Figure 7C: Mild HS activates mitochondrial biogenesis program (*Tfam*)……………………34

Figure 7D: Mild HS activates mitochondrial biogenesis program (*Tfb1m*)……………..……35

Figure 7E: Mild HS activates mitochondrial biogenesis program (*Tfb2m*) ………………..36

Figure 8A: Mild HS increases *Cycs* mRNA expression……………………………...……38

Figure 8B: Mild HS increases *Cox2* mRNA expression…………………………….......39

Figure 8C: Mild HS increases *Cox4* mRNA expression………………………………...40

Figure 8D: Mild HS increases *Glut4* mRNA expression……………………………….41

Figure 9A: Mild HS activates heat shock response (*Hspa1b*)………………………….43

Figure 9B: Mild HS activates heat shock response (*Hspa1b*) …………………………....44

Figure 9C: Mild HS increases HSP70 expression in rat soleus muscle……………………...45

Figure 9D: mtHsp70 mRNA expression after Mild HS…………………………...….46

Figure 10A: Repeated mild HS up-regulates the expression of PGC-1α and oxidative phosphorylation subunits…………………………………………………...…48

Figure 10B: Repeated mild HS up-regulates the mtHsp70 expression…………………...…50

Figure 10C: Repeated mild HS increased mitochondrial DNA copy number……………..….51

Figure 11: Proposed model of mild HS-induced mitochondrial biogenesis correlating with activation of the AMPK-SIRT1- PGC-1α pathway…..…………………………..59

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my advisor, Professor George A. Brooks, for the educational and research opportunities he has provided me as a Ph.D. student. I have benefited in numerous ways from his close supervision throughout my doctoral studies. For me, he continues to be a source of intellectual inspiration and a teacher to whom I can always turn for advice and guidance. I believe the opportunity to have been mentored by such a successful and insightful scientist will benefit me throughout my career.

I thank my dissertation committee (George A. Brooks, Steven L. Lehman, and Sharon E. Fleming) for their invaluable guidance, time and support, and members of my qualifying examination committee (Professors Steven L. Lehman, Robert Dudley, Daniela Kaufer, and Paola S. Timiras) for their guidance in preparing for the topics of the oral examination. I am grateful for the opportunity to have gained knowledge of research methodology and data interpretation from Professor Brooks and the members of his laboratory.

LIST OF ABBREVIATIONS

AMPK: AMP-activated protein kinase

Cox2: cytochrome c oxidase subunit II

Cox4: cytochrome c oxidase subunit IV

Cycs: cytochrome c

Glut4: glucose transporter type 4

Hspa1a: heat shock protein 1A (aka *Hsp72*)

Hspa1b: heat shock protein 1B (aka *Hsp70*)

Ldha: lactate dehydrogenase isoform A

mtHsp70: mitochondrial heat shock protein 70 kDa

NAD: Nicotinamide adenine dinucleotide

Nampt: nicotinamide phosphoribosyltransferase

Nrf1: nuclear respiratory factor 1

Nrf2α: nuclear respiratory factor 2 α-subunit

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PGC-1β: peroxisome proliferator-activated receptor gamma coactivator 1-beta

PRC: peroxisome proliferator-activated receptor gamma coactivator-related protein 1

Rn18s: 18S ribosomal RNA

Sir2: Silent information regulator 2

SIRT1: sirtuin 1

Tfam: transcription factor A, mitochondrial

Tfb1m: transcription factor B1, mitochondrial

Tfb2m: transcription factor B2, mitochondria

CHAPTER 1

INTRODUCTION

A. Review of the literature

Maintenance of Body Temperature

Temperature directly or indirectly affects essentially every aspect of an organism's physiology. It is well known that most species perform best in a fairly narrow temperature range, which is also associated with optimal enzyme functions (51). Endotherms, including birds and mammals, have evolved adaptations to maintain a constant core body temperature. In addition to behavior modification and proper insulation, endotherms produce heat by thermogenesis involving different mechanisms. Shivering thermogenesis is a common strategy that works for short-term cold exposure. Sympathetic neural stimulation activates the actomyosin ATPases of skeletal muscle, the unsynchronized muscle contraction results in heat generation but not gross movement (55). During prolonged cold stress, nonshivering thermogenesis begins to kick in. Uncoupling proteins such as thermogenin in brown adipose tissue generate heat by mitochondrial proton leak (33, 55). Excess heat in mammals is dissipated by evaporative means (panting and sweating) or non-evaporative means (conduction, convection and radiation) (7, 13).

Exercise-induced Stresses

Endurance exercise imposes a combination of stresses such as changes in calcium concentration, mechanical strain, alternations in the ADP to ATP ratio, hypoxic stress and loosening of mitochondrial respiratory control on skeletal muscle (8, 44) (Table 1). Numerous studies have focused on how these stresses contribute to exercise-induced adaptations. For example, increased muscle use results in increased levels of intracellular free calcium concentration and calcium-activated calcineurin plays a crucial role in determining muscle fiber types (14). Increased heat production is also a byproduct of endurance exercise, and can also be counted among the stresses imposed upon skeletal muscle by physical exercise. However, the direct effects of mild HS on mitochondrial biogenesis have not been well characterized in past studies.

Efficiency of Energy Conversion and Muscle Temperature during Exericse

Muscle tissue is capable of directly transforming the chemical energy derived from

nourishment into mechanical work. Measured by the ratio of work performed to energy cost as calculated from oxygen uptake, the efficiency of bicycling humans is 20% to 30% (21, 73). The efficiency is determined by both the efficiency of ATP generation (phosphorylative coupling efficiency) and the efficiency of ATP use (contracting coupling efficiency). Typical values of phosphorylative coupling efficiency reported are about 60% and contracting coupling efficiency about 50% (21, 73). The energy conversion efficiency is even lower (12%) if determined by the ratio of work performed to the total enthalpy produced (i.e., heat plus work) using isolated mouse muscles (67). Intriguingly, human muscle may be more efficient than muscles of small mammals. A recent study reported that the contraction coupling efficiency of the human first dorsal interosseous (FDI) muscle is 68% (41). The higher efficiency of human muscle may be accounted for by higher efficiency in mechanics (56) and in activation (31).

During vigorous exercise, the energy cost can increase by 10- to 20-fold compared to the resting metabolic rate, while more than 70% of the energy derived from the oxidation of metabolic fuels is dissipated as heat (46). The accumulation of heat leads to an increase in both core and shell temperature in the human body. For example, in humans the temperature of exercising muscles can rise to values as high as 40°C after exhaustive exercise or during submaximal exercise on a bicycle ergometer (62, 63), while the average gastrointestinal temperature can rise to values as high as 39.3°C after running for 45 minutes outdoors (46), and in rats made to exercise by treadmill running even higher muscle temperatures have been observed (9). The heat generated during endurance exercise must be effectively transported to the skin and dissipated to the environment in order to prevent heat injury. However, the mechanisms involved in heat dissipation are not always enough; heat stroke is currently the third leading cause of death in athletes in the United States (36).

Heat Dissipation and Evolution of Endurance Running in Human

Heat dissipation is an evolutionarily important issue, in particular for humans. Endurance running is believed to be a derived trait of the genus Homo, originating about 2 million years ago in response to the challenges of life in an open tropical savanna (6). Man's upright posture and bipedal gait make the energetic cost of transport relatively high in comparison with that for other mammals and running birds (13). To be adapted to sustained levels of aerobic exercise and persistence hunting, endogenous heat generated by muscular activity was a serious problem that needed to be solved. Therefore, humans developed the most sophisticated of all mammalian cooling mechanisms including a narrow, elongated body form,

the lack of body hair, the tendency for mouth breathing, well-developed sweat glands as well as cranial cooling systems that allow the brain to remain cooler than the rest of the body (6, 13).

Beneficial Effects of Mild Heat Stress

Although severe heat injury can be lethal, several beneficial effects of mild HS - heating within physiological limits - have been reported among several different species.

1. Mild HS and Metabolism

Hooper reported that improvement of insulin sensitivity and lower blood glucose levels in patients with type 2 diabetes were observed after sitting in a hot tub (temperature ranged from 37.8°C to 41.0°C) for 30 minutes a day, six days a week, for three weeks (35). The mechanisms behind this effect were unclear.

A recent study reported that an elevation in heat shock protein 72 (Hsp72) levels protected against diet- or obesity-induced insulin resistance and hyperglycemia in mice (15). The elevation in Hsp72 was achieved using weekly heat shock therapy at 41.5C for 15 min for 16 weeks, transgenic overexpression, and pharmacologic means. Another study showed that weekly heat shock therapy (at 41 and 41.5°C for 20 min for 12 weeks) could improve high-fat diet–induced skeletal muscle insulin resistance in rats and increased oxygen consumption and fatty acid oxidation in L6 cells (26). These beneficial effects correlated with preventing phosphorylation of c-jun amino terminal kinase (JNK) and inhibitor of κB kinase (IKK), which impair the insulin signaling pathway. However, the authors did not provide a mechanism for how heat shock proteins regulate JNK and IKK activation.

2. Mild HS and Life Span

Several studies have reported a connection between transient heat treatments and extended life span at normal temperatures. *Caenorhabditis elegans* stocks are typically maintained at 20°C. A single bout of HS by exposure to a sublethal temperature of 30°C for 3-24 hr induced thermotolerance and led to a statistically significant increase in the life span of wild-type C. elegans by 14% (48). *Drosophila melanogaster* stocks were typically

maintained at 24 °C. A brief HS by exposure of 4-day-old experimental flies to 36 °C for 70 min extended life span by an average of 2 days (42). The HS also increased thermotolerance to subsequent thermal stress. The yeast *Saccharomyces cerevisiae* cells were cultured at 30°C in media. Two brief sublethal HS at 37°C for 2hr early on in life resulted in a significant extension in mean life span by 10% (66). Similar to that found in *C. elegans* and *D. melanogaster*, thermotolerance was also induced. In summary mild HS is one of the two well-known environmental treatments that extend life span in animals. (The other one is calorie restriction.)

Cytoprotective Heat Shock Response

One of the most prominent mechanisms to rapidly cope with stresses is the heat shock response (32, 47). The heat shock response is almost ubiquitous across all life forms and is induced at temperatures a few degrees above the normal physiological range for most species (55). To cope with stresses, cells need to synthesize several critical heat shock proteins that can be used to restore structures of reversibly damaged proteins while degrading irreversibly damaged proteins as well as synthesizing new proteins (32, 47). All of these events are highly ATP-costly processes and force cells into a reduced energy status.

Key Energy-sensing Molecules and Energy Metabolism

1. AMP-activated Protein Kinase (AMPK)

AMPK is a key energy-sensing molecule which maintains whole body energy homeostasis in response to reduced cellular energy status (29) (Figure 1). Because of the reaction catalyzed by adenylate kinase (2ADP → ATP + AMP), the AMP:ATP ratio is a sensitive indicator of cellular energy status. AMPK is allosterically activated by AMP. In addition, the phosphorylation of the threonine 172 (T172) residue by upstream kinases is required for AMPK activity. Activated AMPK switches off biosynthetic pathways to reserve ATP for more essential processes and switches on catabolic pathways to boost ATP production by upregulating oxidative metabolism and mitochondrial biogenesis in skeletal muscle (30). Using stable isotope tracers and a synthetic peptide substrate for AMPK, a previous study showed that HS at 45°C raised AMP:ATP ratio by 13 times, which, in turn, activated AMPK immediately in isolated rat hepatocytes (16).

2. Sirtuin 1 (SIRT1)

Silent information regulator 2 (Sir2) proteins, or sirtuins, have been implicated as critical regulators for aging and longevity in experimental model organisms including yeast, worms and flies (5). Sirtuins are class-III histone deacetylases which can deacetylate non-histone proteins (45). Sirtuins require nicotinamide adenine dinucleotide (NAD) as an essential cofactor for their deacetylase activity (38). The dependency on NAD links sirtuin activity to the energy state and metabolic response of an organism (65). SIRT1, the closest homologue of Sir2 in mammals, is an important regulator of metabolism, cancer, aging and possibility longevity in mammals (37, 45) (Figure 2). Recently, an emerging body of evidence suggests that SIRT1 works closely with AMPK to control cellular energy expenditure (20, 61).

3. Peroxisome Proliferator-activated Receptor Gamma Coactivator-1α (PGC-1α)

In response to external stimuli, the transcriptional coactivator PGC-1 α coordinates a variety of transcription factors and nuclear receptors in the control of cellular energy metabolic pathways in different tissues (28) (Figure 3). Because PGC-1α induces mitochondrial biogenesis by upregulating and interacting with the nuclear respiratory factor 1 and 2 (NRF1 and NRF2) and the estrogen-related receptor 1 (52, 75), it has been described as a master regulator of mitochondrial biogenesis. The expression and activity of PGC-1α are regulated by a variety of upstream cellular signaling pathways. Prior studies indicate that PGC-1 α expression in skeletal muscle was very responsive to both short-term exercise and endurance training (3, 25, 68, 69).

AMPK-SIRT1-PGC-1α pathway and Mitochondrial Biogenesis

AMPK and SIRT1 act in cooperation with PGC-1α to regulate energy homeostasis in response to environmental stimuli and nutritional signals (11, 12, 75). AMPK and SIRT1 directly activate PGC-1α through phosphorylation and deacetylation, respectively (39, 60). AMPK and SIRT1 also increase the expression of mRNAs encoding PGC-1α (2, 39). Together with mitochondrial transcriptional factors and genes of mitochondrial components, the AMPK-SIRT1-PGC-1α pathway is one of the major pathways inducing mitochondrial biogenesis (23, 75) (Figure 4).

Mitochondrial biogenesis entails mitochondrial DNA (mtDNA) replication and transcription, as well as the synthesis and import of proteins into the mitochondrial reticulum

(40, 43). Because the oxidative phosphorylation system, comprised of five multi-subunit enzyme complexes, is located in the mitochondrial inner membrane (64), the physiological significance of mitochondrial biogenesis lies in the enhancement of oxidative phosphorylation, which ultimately results in a net ATP gain of more than 15 times the amount of ATP produced by glycolysis (1).

B. Hypothesis

We believe that the beneficial effects of mild HS, such as improved insulin sensitivity, may be associated with mitochondrial biogenesis. We hypothesize that the reduced level of cellular energy induced by mild HS can activate AMPK and SIRT1, both of which further upregulate PGC-1α and the downstream mitochondrial biogenesis program. C2C12 are a widely used model system that mimics what happens in vivo. To test this hypothesis, we used C2C12 myotubes to study the effect of acute and repeated mild HS on mitochondrial biogenesis by measuring activation of the AMPK-SIRT1-PGC-1α pathway, as well as the levels of transcription factors involved in mitochondrial biogenesis and mitochondrial components such as mitochondrial DNA copy number.

Table 1

Signal related to endurance exercise	Molecular events	
Tonic motor nerve activity		
↗ Calcineurin activity	Nuclear translocation of NFAT	
	↗ MEF2 transcriptional activity	
Changes in $[Ca^{2+}]i$	↗ p38 activity	
	↗ ERK1/2 activity	
	↗ PKC activity	
	↗ CaMKII activity	
Mechanical stress		
Dynamic exercise	↗ ERK1/2 activity	After exhaustive muscle activity
	↗ p38 activity	Mainly in untrained muscles
Static stretch or eccentric contraction	↗ JNK, ERK1/2 and p38 activities	Major changes
Isometric contraction	↗ ERK1/2 activity	Response to mechanical strain
Metabolic signals		
Changes in ADP to ATP ratio	↗ AMPK activity	PGC-1α gene induction
Hypoxic stress	Physiological activation of HIF-1	Expression of hypoxia-responsive genes

Table 1 Main effects of physical and metabolic signals associated with endurance exercise. Table adapted from Koulmann and Bigard (2006).

Figure 1

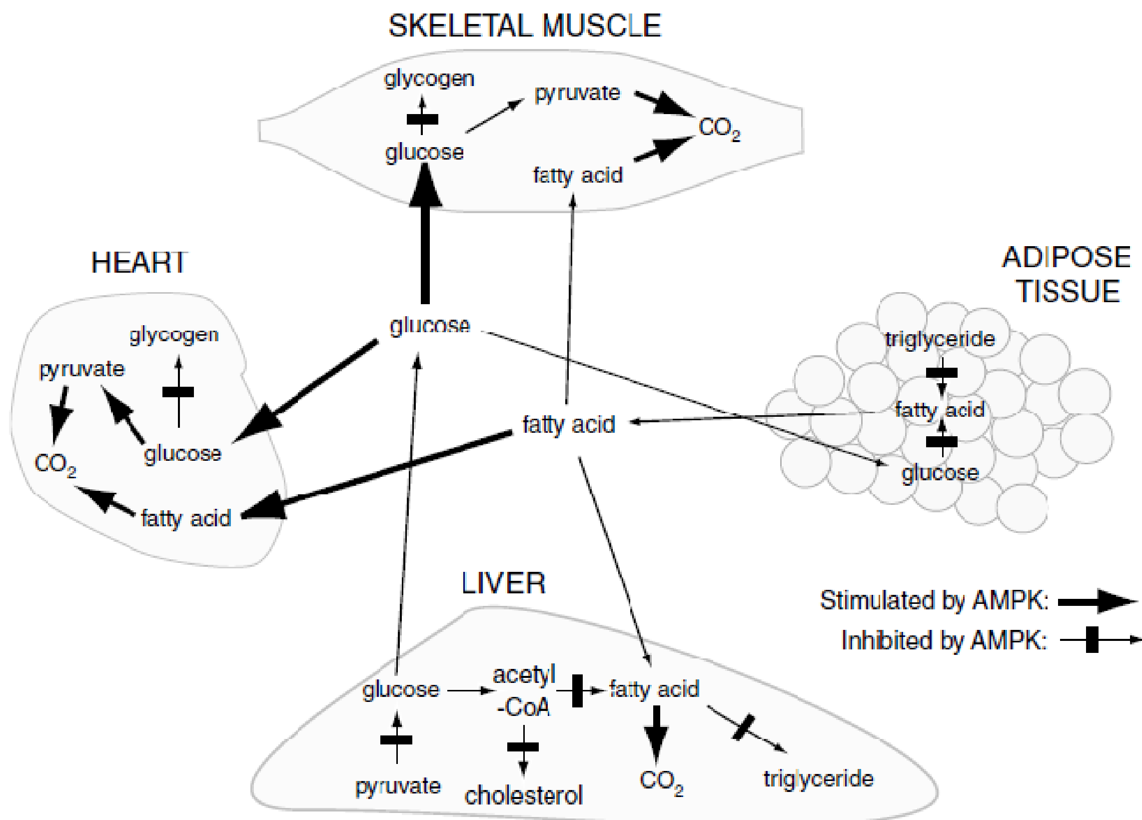


Figure 1: Metabolic changes induced by AMPK in a variety of tissues. The diagram summarizes the metabolic changes known to be induced by AMPK activation in the liver, heart, skeletal muscle and adipose tissue of mammals in response to reduced cellular energy status. Figure adapted from Hardie (2008).

Figure 2

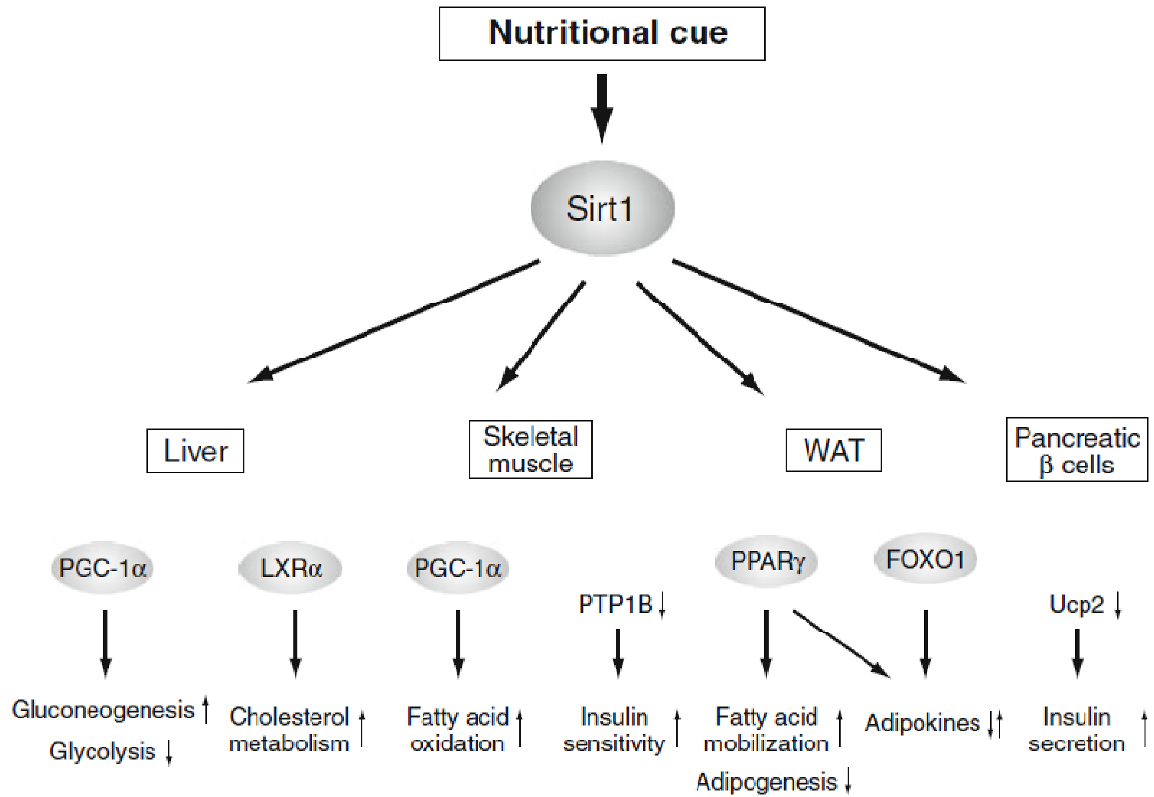


Figure 2: SIRT1 coordinates metabolic responses to nutritional availability in various tissues. By regulating PGC-1α or directly interacting with transcription factors, SIRT1 can modulate gene expression profiles in target tissues such as liver, skeletal muscle, white adipose tissue and pancreatic beta cells. Figure adapted from Imai (2006).

Figure 3

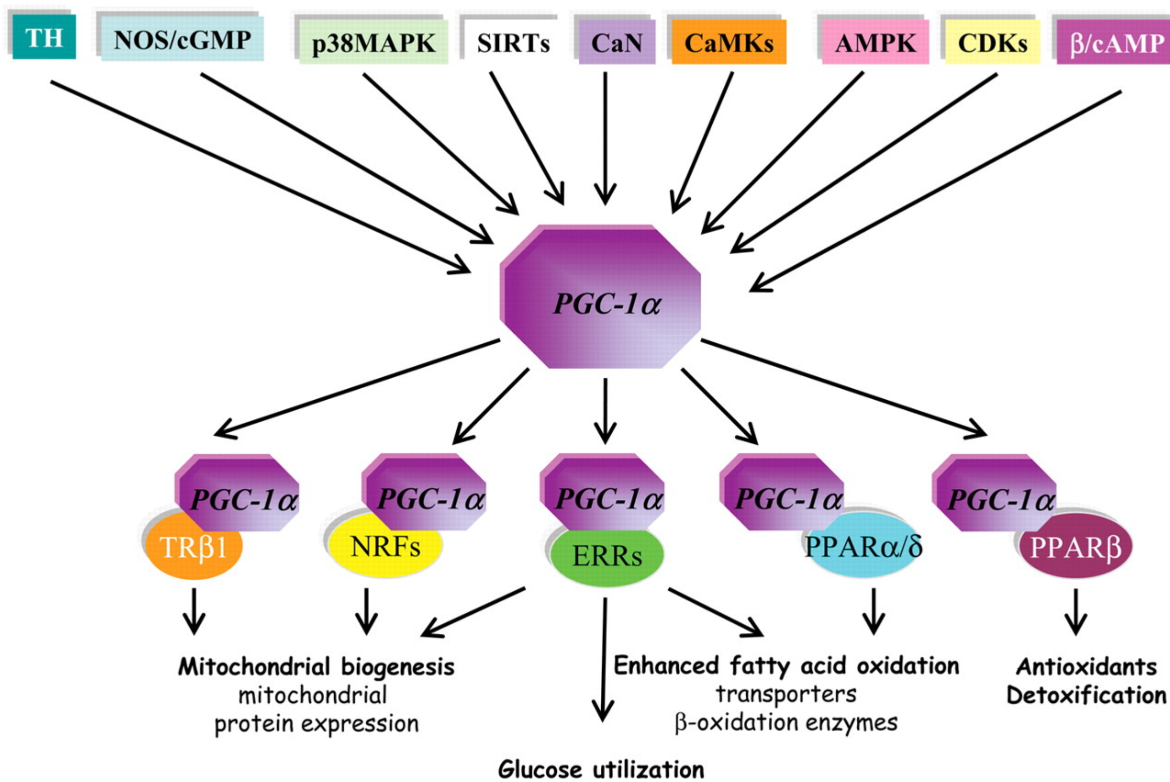


Figure 3: PGC1α regulatory cascade. The diagram summarizes the regulatory network orchestrated by PGC-1α and its interactions with some of its target transcription factors involved in metabolic regulation. Figure adapted from Ventura-Clapier et al. (2008)

Figure 4

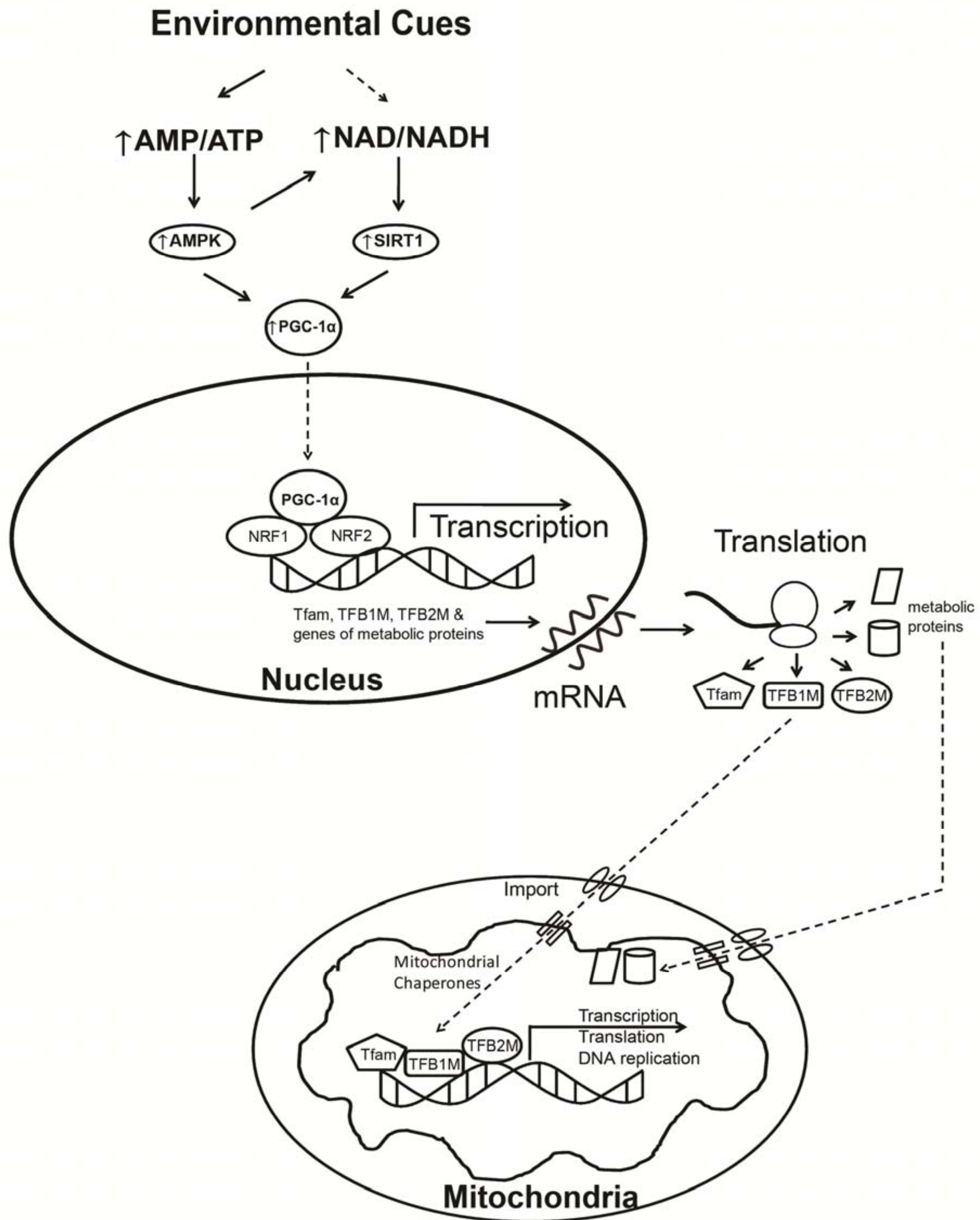


Figure 4: Schematic representation of mitochondrial biogenesis by the AMPK-SIRT1-PGC-1α pathway. The effects of PGC-1α on mitochondrial biogenesis are mediated through its ability to both upregulate expression of NRF1 and NRF2 as well as co-activate both nuclear respiratory factors leading to transcription of nuclear-encoded metabolic proteins and of the mitochondrial transcription factors, including TFAM, TFB1M and TFB2M. Mitochondrial transcription factors activate transcription and replication of the mitochondrial genome. Nuclear-encoded metabolic proteins are imported into mitochondria through membrane transport machinery. Nuclear- and mitochondria-encoded subunits of the respiratory chain are then assembled.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture

Mouse C2C12 myoblast cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) (4.5 g glucose/L) in the presence of 10% fetal bovine serum. Differentiation of C2C12 myoblasts into myotubes was induced with media containing 2% horse serum for five days. The cells were grown in a humidified 37°C incubator with 5% CO_2 in air.

Mild heat stress exposure

The American College of Sports Medicine (ACSM) recommends moderate-intensity cardiorespiratory exercise training for ≥30 min/d on ≥5 d/wk for most adults (22). Therefore, we chose 1hr HS for a single bout or 1hr for 5 days as the treatment. For mild HS, the culture temperature was set at 40°C. The duration of mild HS was maintained at one hour (hr) for each bout. The frequency was either a single bout on the 5th day of C2C12 myotube differentiation or one bout per day for 5 days since the start of differentiation. After final treatment cells were harvested for subsequent assays.

Total protein extraction

C2C12 myotubes were washed with 1x phosphate buffered saline (PBS), scraped, centrifuged and resuspended in sodium dodecyl sulfate (SDS) lysis buffer (50mM Tris-HCl pH 6.8, 1% SDS and 10% Glycerol). After brief sonication to break up genomic DNA, an aliquot of the cell lysates was reserved for subsequent protein concentration determinations (BCA assay, Thermo Fisher), and the remainder was supplemented with β-mercaptoethanol.

Western blotting

Total cell lysates of C2C12 myotubes were separated by SDS-polyacrylamide gel electrophoresis and transferred to Hybond-C Extra nitrocellulose membranes (Amersham Biosciences), and probed with antibodies against AMPK (Sigma), phosphorylated AMPK (Cell Signaling), SIRT1 (Abcam), PGC-1α (Millipore), Nampt (Bethyl), Hsp70 (Invitrogen), mitochondrial HSP70 (Thermo) and α-Tubulin (Sigma) as well as with OXPHOS Rodent WB Antibody Cocktail (MitoSciences), which recognizes Complex I subunit NDUFB8, Complex II subunit 30 kDa, Complex III subunit Core 2, Complex IV subunit I and ATP synthase subunit α. For normalization purposes, blots were also probed with an antibody to Histone H3 (Upstate). Blots were then developed using Amersham ECL Western Blotting Detection (GE Healthcare). The band intensity was quantified with Photoshop and ImageJ

software.

RNA Isolation and cDNA Synthesis

Total RNA in harvested C2C12 myotubes was isolated by RiboPure kits (Ambion) according to the manufacturer's instructions. Isolated RNA was reverse transcribed to cDNA using random hexamer primers and SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions.

Real-Time Quantitative PCR

Real time quantitative PCR (qPCR) was performed using 2X HotSybr Real-time PCR Kit (Mclab) and iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Sequences of specific primers are listed in Table 2. The threshold cycle (Ct) indicates the fractional cycle number at which the amount of amplified copies reaches a fixed threshold..The data from qPCR were analyzed with delta-delta Ct (ΔΔCt) method using 18S ribosomal RNA as the internal control gene. Each delta Ct (ΔCt) value was determined by subtracting 18S ribosomal RNA Ct value from the target gene Ct value. The ΔΔCt was calculated by subtracting the ΔCt value of the 37°C control from the ΔCt value of the heat-stressed sample. $2^{-\Delta\Delta Ct}$ represented the average relative amount of mRNA for each target gene.

Reporter gene assays

C2C12 myocytes were transfected with 1 ug of PGL3-PGC-1α 2-kb promoter-firefly luciferase reporter vector (Addgene) (27) and 0.2 ug of CMV Renilla luciferase vector as the control vector in 6-well plates at 90% confluence using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Cells were left for 5h with the DNA-Lipofectamine mix. Then, the medium was removed and replaced by differentiation medium for 36h before being treated with mild HS for 1hr. After a 24-hrrecovery period, the cultures were resuspended into 250 ul of passive lysis buffer from the Dual Luciferase Reporter Assay system (Promega) and 20 ul of the cell lysates were used for determining Renilla and firefly luciferase activities with the Promega kit mentioned above.

Mitochondrial DNA Quantification

Total cellular DNA in C2C12 myotubes was extracted using Blood & Cell Culture DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA pellets were

resuspended in Tris-EDTA (TE) buffer (1 mM EDTA, 10 mM Tris, pH 8.0) prior to qPCR analysis. qPCR was performed for mtDNA-encoded cytochrome c oxidase subunit II (*Cox2*) (primers: forward 5'-GCCGACTAAATCAAGCAACA-3', reverse 5'-CAATGGGCATAAAGCTATGG -3') (77) and nucleus-encoded 18S ribosomal RNA genes (primers: forward 5'- TAGAGGGACAAGTGGCGTTC-3', reverse 5'-CGCTGAGCCAGTCAGTGT-3') (4). The ratio of *Cox2*DNA copies to 18S rRNA represents the relative mitochondrial copy number.

Statistical analysis

Statistical significance of temperature-induced differences was evaluated by Student's *t*-test assuming equal variance, and a value of $P < 0.05$ was considered significant. Results are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using Excel 2007 software (Microsoft).

Table 2 Primer sequences used for real-time quantitative PCR

Primers	Strand	Sequence 5'-> 3'
Hspa1a	Forward	AGATATGTGGCCTTGAGGACTGTCATTATTTC
	Reverse	CAAATCACATCAGCGGGGCAGTGCTGAATTG
Hspa1b	Forward	TGCTTGGGCACCGATTACTGTCAAGG
	Reverse	GGCAGCTAGACTATATGTCTTCCCAGGCTACTG
Sirt1	Forward	GACGATGACAGAACGTCACAC
	Reverse	CGAGGATCGGTGCCAATCA
PGC-1α	Forward	AAGTGTGGAACTCTCTGGAACTG
	Reverse	GGGTTATCTTGGTTGGCTTTATG
PGC-1β	Forward	GGCAGGTTCAACCCCGA
	Reverse	CTTGCTAACATCACAGAGGATATCTTG
PRC	Forward	CCAAAAAGGATGCCTGCCCTA
	Reverse	GTAGCCGTGCATGGGAGTG
Nrf1	Forward	TCT CAC CCT CCA AAC CCA AC
	Reverse	CCC GAC CTG TGG AAT ACT TG
Nrf2α	Forward	CTCCCGCTACACCGACTAC
	Reverse	TCTGACCATTGTTTCCTGTTCTG
Tfam	Forward	CAT TTA TGT ATC TGA AAG CTTCC
	Reverse	CTC TTC CCA AGA CTT CAT TTC
Tfb1m	Forward	AAGATGGCCCTTTCGTTTATGG
	Reverse	GACTGTGCTGTTTGCTTCCTG
Tfb2m	Forward	CCAAAACCCATCCCGTCAAAT
	Reverse	AAGGGCTCCAAATGTGGAATAAA
Cycs	Forward	GCAAGCATAAGACTGGACCAAA
	Reverse	TTGTTGGCATCTGTGTAAGAGAATC
Cox2	Forward	TGAAGACGTCCTCCACTCATGA
	Reverse	GCCTGGGATGGCATCAGTT
Cox4	Forward	ACCAAGCGAATGCTGGACAT
	Reverse	GGCGGAGAAGCCCTGAA
Glut4	Forward	TGTGGCCTTCTTTGAGATTGG
	Reverse	CCCATGCCGACAATGAAGTT
Ldha	Forward	TGCCTACGAGGTGATCAAGCT
	Reverse	ATGCACCCGCCTAAGGTTCTT
Rn18s	Forward	CGCCGCTAGAGGTGAAATTCT
	Reverse	CGAACCTCCGACTTTCGTTCT

CHAPTER 3

RESULTS

A. Mild heat stress increases AMPK activity and SIRT1 expression

To investigate the direct cellular effects of mild HS on muscle cells, we incubated the C2C12 myotubes at 40°C for one hr. Cells were harvested immediately after mild HS. The AMPK protein levels and phosphorylation status at the T172 residue were determined by Western blotting. The degree of AMPK T172 phosphorylation was used as a measure of AMPK activation (30). It is evident from Figure 5A that, immediately after mild HS, a significant increase in AMPK T172 phosphorylation (1.6-fold; $P < 0.05$) was observed while the AMPK protein levels remained unchanged. These results are interpreted to mean that mild HS rapidly enhances AMPK activity.

AMPK is known to regulate SIRT1 expression (76). Therefore, we measured SIRT1 expression after mild HS. Our data showed that SIRT1 protein levels remained unchanged immediately after mild HS (Figure 5A). However, 24 hr after mild HS, SIRT1 protein levels increased by 2.04-fold ($P < 0.05$) (Figure 5B). We also found that *Sirt1* mRNA levels increased 24 hr after mild HS (1.51-fold, $P < 0.05$) (Figure 5C).

NAD is required in several important biological functions. In particular, NAD is an obligate cofactor for the deacetylase activity of SIRT1 (38). Nicotinamide phosphoribosyltransferase (Nampt) is responsible for the rate-limiting step in the conversion of nicotinamide to nicotinamide mononucleotide in the NAD biosynthetic pathway in mammals (59). Therefore, we measured Nampt expression after mild HS. Our data showed that 24 hr after mild HS, Nampt protein levels significantly increased (1.50-fold $P < 0.05$) (Figure 5D). In summary, a single bout of mild stress resulted in a rapid increase of AMPK activity followed by an increase of Nampt and SIRT1 expression.

Figure 5A

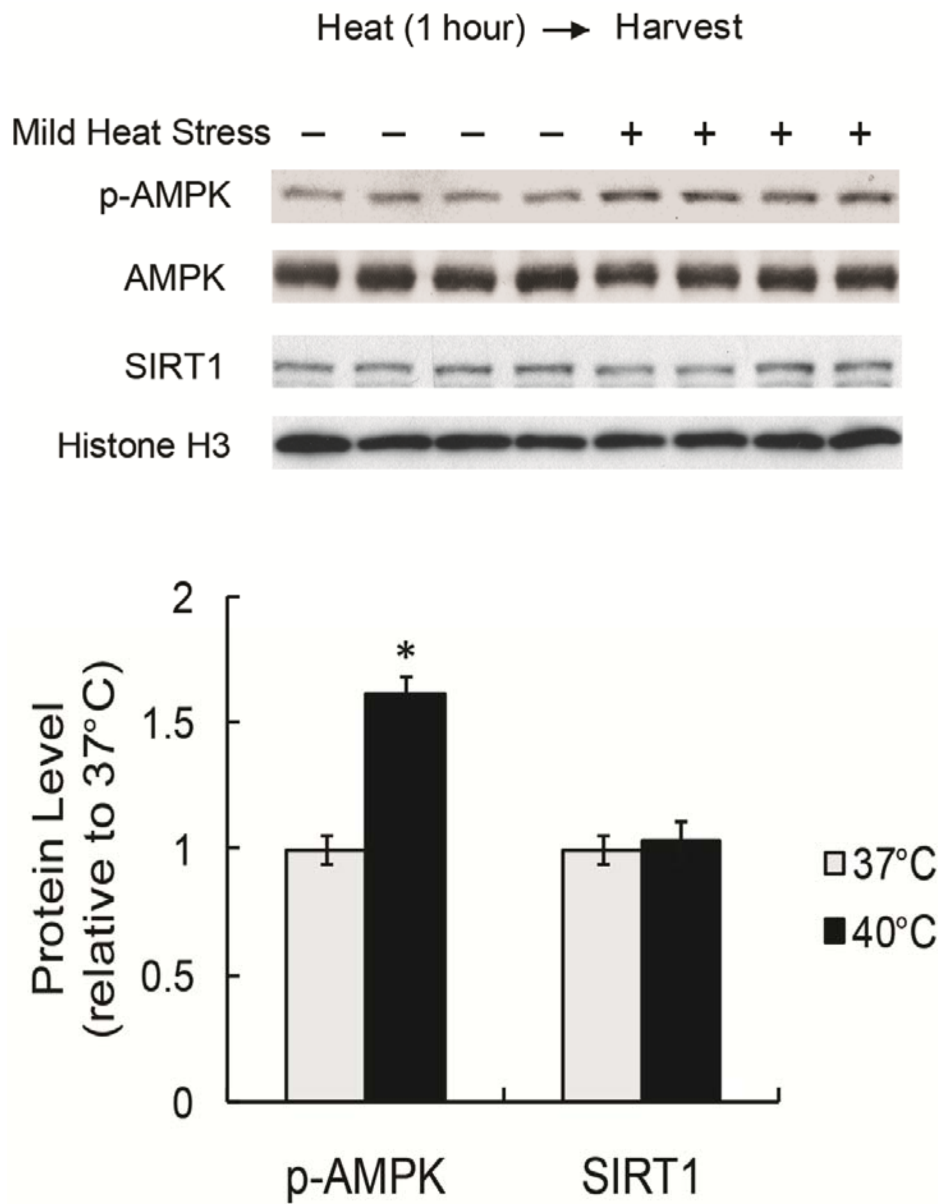


Figure 5A: Mild HS increases AMPK activity. C2C12 myotubes were exposed to 1-hr mild HS and harvested immediately for Western blot. Results were normalized to Histone H3. Quantification of band intensity is shown below the Western blot. Results are given as the means ± SEM from four independent trials.*$P < 0.05$ compared to the control.

Figure 5B

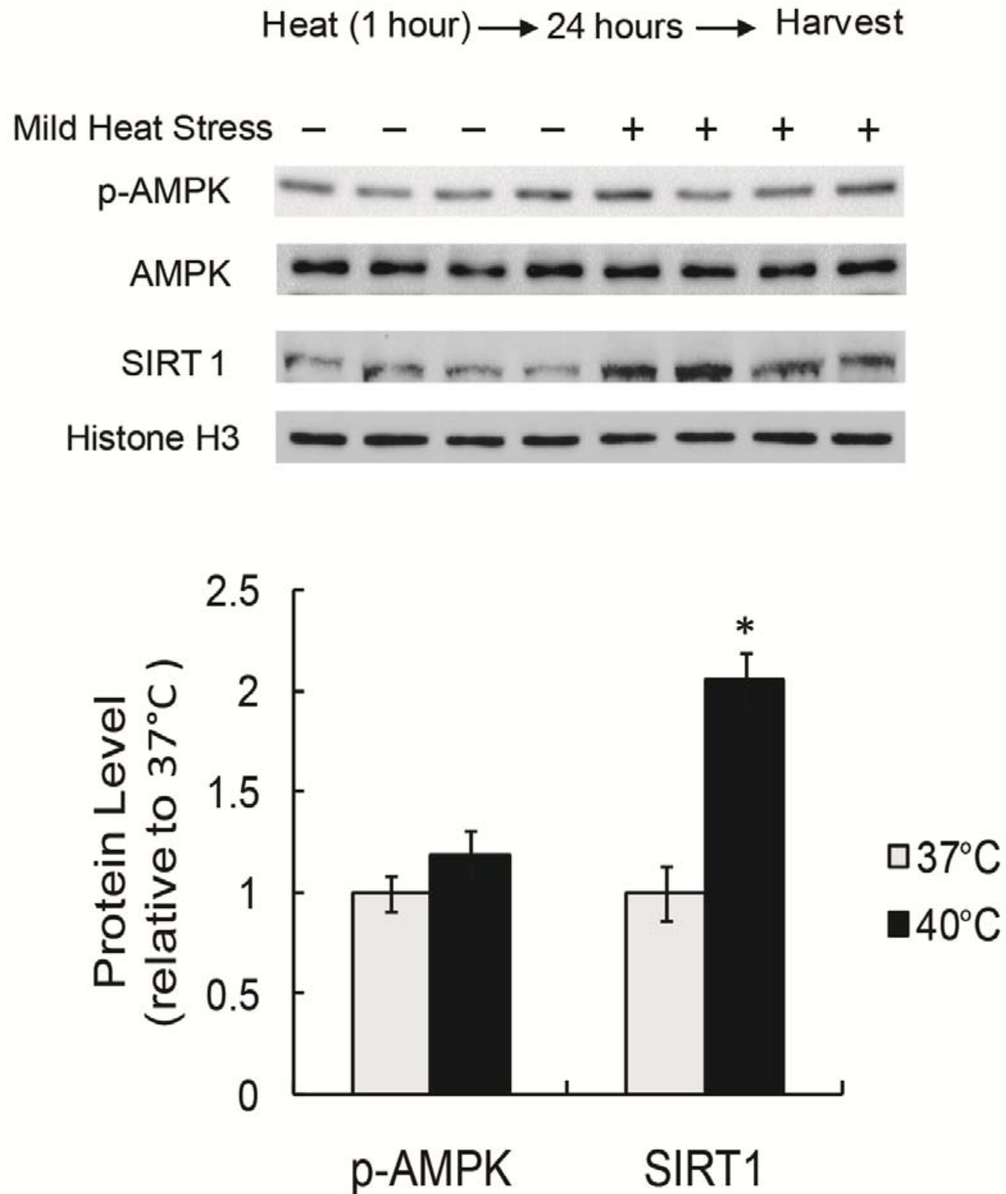


Figure 5B: Mild HS increases SIRT1 expression. C2C12 myotubes were exposed to 1-hr mild HS (40°C) and then returned to the normal culture environment (37°C). After a 24-hr recovery period, cells were harvested for Western blot. Results were normalized to Histone H3. Quantification of band intensity is shown below the Western blot. Results are given as the means ± SEM from four independent trials.**P* < 0.05 compared to the control.

Figure 5C

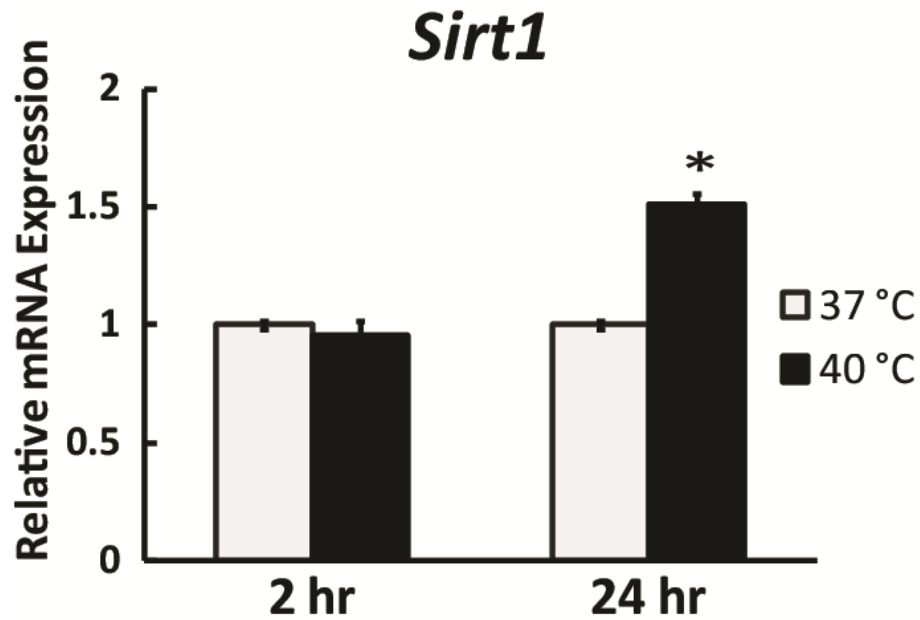


Figure 5C: Mild HS increases *Sirt1* mRNA expression. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). The mRNA levels of *Sirt1* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to control.

Figure 5D

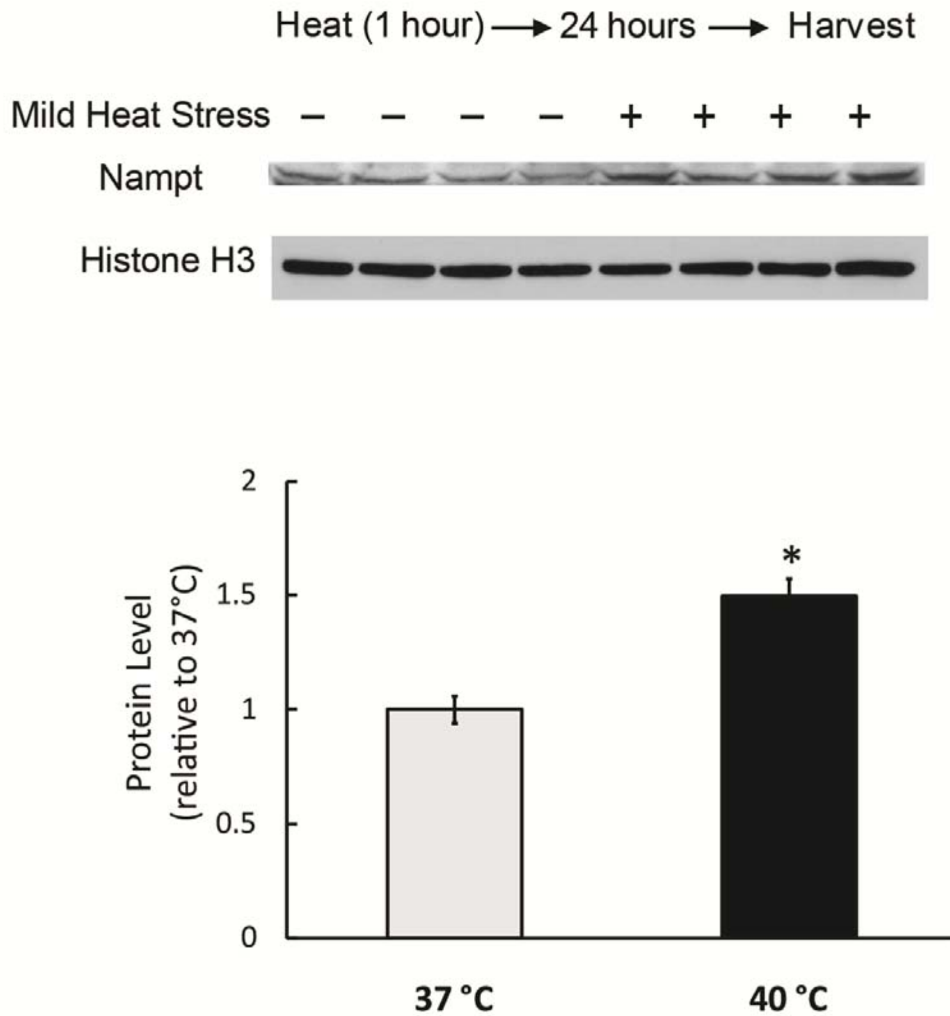


Figure 5D: Mild HS increases Nampt expression. C2C12 myotubes were exposed to 1-hr mild HS (40°C) and then returned to the normal culture environment (37°C). After a 24-hr recovery period, cells were harvested for Western blot. Results were normalized to Histone H3. Quantification of band intensity is shown below the Western blot. Results are given as the means ± SEM from four independent trials.*P < 0.05 compared to the control.

B. Mild heat stress increases PGC-1α transcription

PGC-1α is a well studied transcriptional target of both SIRT1 and AMPK (2, 39). In our studies of its expression levels after mild HS, no significant change in the mRNA levels of PGC-1α was observed 2 hr after mild HS while an increase of 1.36-fold ($P < 0.05$) was seen 24 hr later (Figure 6A). In contrast, the mRNA levels of PGC-1β and PRC, the other two members of PGC-1 family transcription co-activators, remained constant at both 2 hr and 24 hr after mild HS (Figures 6B-C).

To examine whether mild HS increases PGC-1α expression at the transcription level, a reporter construct expressing luciferase under the control of 2-kb mouse PGC-1α promoter was transfected into C2C12 myocytes. Subsequently, the reporter luciferase activity in response to mild heat stress was determined. As shown in Figure 6D, luciferase activity increased by 3.46-fold ($P < 0.05$) 24 hr after mild HS. In summary, our data indicate that mild HS is sufficient to increase PGC-1α promoter activity and modulate PGC-1α transcription in C2C12 myotubes.

Figure 6A

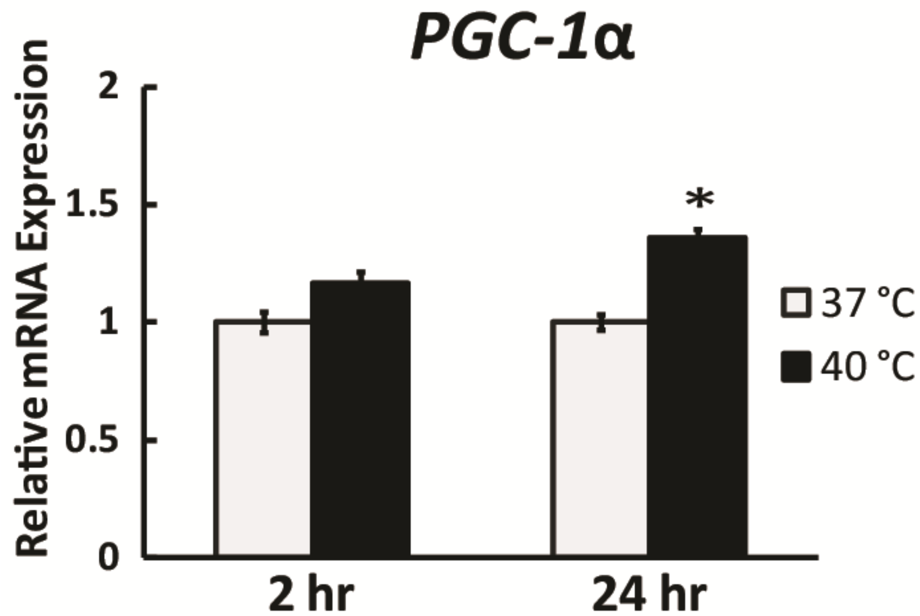


Figure 6A: Mild HS increases *PGC-1α* mRNA expression. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). The mRNA levels of *PGC-1α* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to control.

Figure 6B

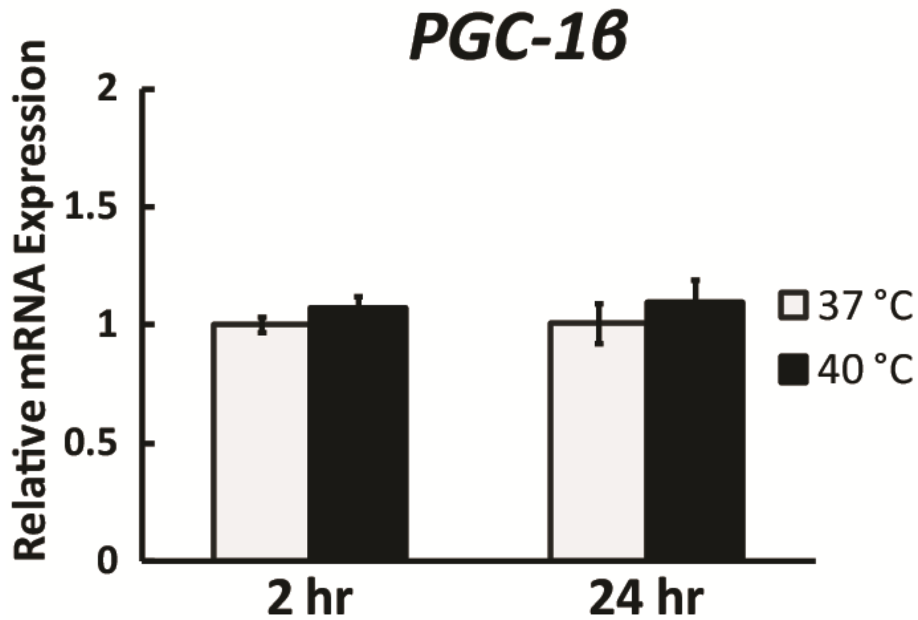


Figure 6B: *PGC-1β* mRNA expression after Mild HS. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). The mRNA levels of *PGC-1β* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. No significant difference detected.

Figure 6C

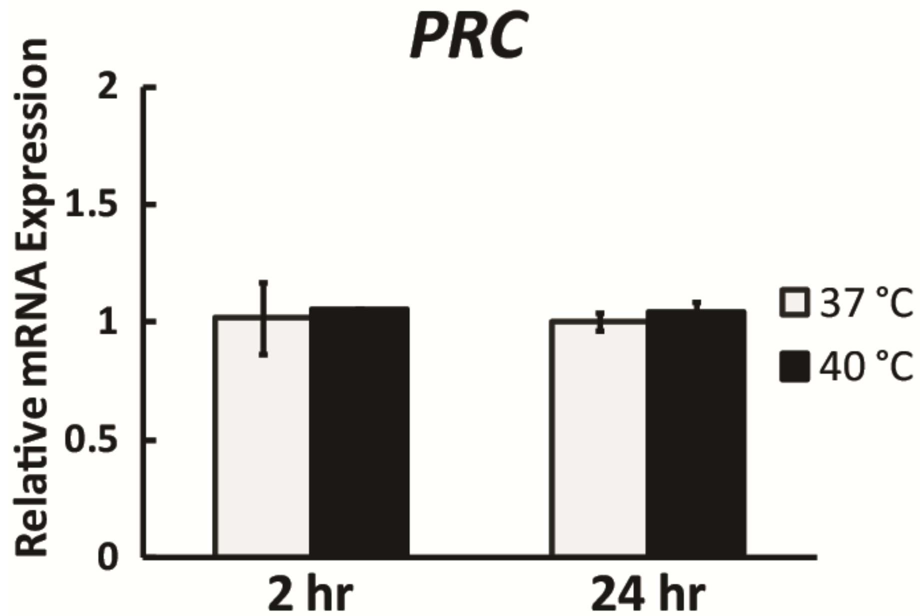


Figure 6C: *PRC* mRNA expression after Mild HS. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). The mRNA levels of *PRC* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. No significant difference detected.

Figure 6D

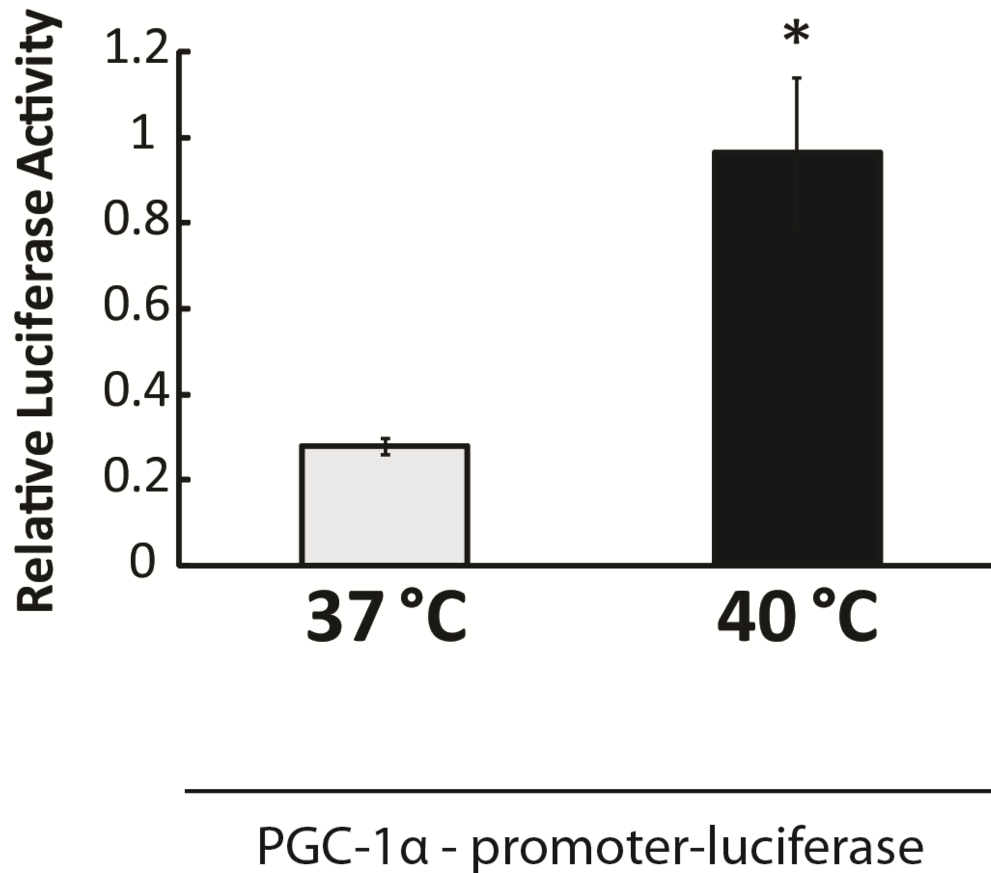


Figure 6D: Mild HS increases *PGC-1α* transcription. C2C12 myoblasts were transfected with PGL3-PGC-1α 2-kb promoter-firefly luciferase reporter and a CMV Renilla luciferase plasmid. After differentiation, the cells were treated with 1-hr mild HS and then returned to the normal culture environment (37°C). After a 24-hr recovery period, cells were harvested for luciferase assay. The data show the ratio of firefly to Renilla luciferase luminescence, which represents the normalized promoter activity of PGC-1αResults are given as the means ± SEM from 5 independent trials.*$P < 0.05$ compared to the control.

C. Mild heat stress activates mitochondrial biogenesis program

To test whether mitochondrial biogenesis program is upregulated in response to mild heat stress, a series of real-time qPCR assays was conducted to compare the expression of downstream genes of AMPK-SIRT1-PGC-1α pathway. 2 hr or 24 hr after one-hr of mild HS, C2C12 myotubes were harvested for qPCR to compare the mRNA levels of genes involved in the mitochondrial biogenesis program.

We first analyzed NRF1 and NRF2, the transcription factors necessary for the coordination of nuclear-mitochondrial gene expression required for mitochondrial biogenesis. The effects of PGC-1α on mitochondrial biogenesis are mediated through its ability to both upregulate expression of NRF1 and NRF2 as well as co-activate both nuclear respiratory factors (75). Similar to PGC-1α, the mRNA levels of both *Nrf1* and *Nrf2α* remained constant 2 hr after mild HS while a significant increase was observed after 24 hr (1.35-fold, $P < 0.05$ and 1.46-fold, $P < 0.05$ respectively) (Figures 7A and 7B).

Mitochondrial DNA replication and transcription are coupled events executed by unique enzyme systems. This process is regulated by mitochondrial transcription factors, including TFAM, TFB1M and TFB2M (18). After nuclear translocation and co-activation by PGC-1α, NRF1 and NRF2 bind and activate the promoters of *Tfam*, *Tfb1m* and *Tfb2m* (23). Because the mRNA levels of both *Nrf1* and *Nrf2α* increased by mild HS, we subsequently measured the mRNA levels of *Tfam*, *Tfb1m* and *Tfb2m*. Two hr after mild HS, the mRNA levels of these three mitochondrial transcription factors remained constant. However, 24 hr after treatment, a significant increase was observed in the levels of all three transcription factors (1.55-fold, $P < 0.05$, 1.27-fold, $P < 0.05$ and 1.77-fold, $P < 0.05$ respectively) (Figures 7C-E). In summary, these data can be interpreted to mean that the mitochondrial biogenesis program is upregulated by mild heat stress.

Figure 7A

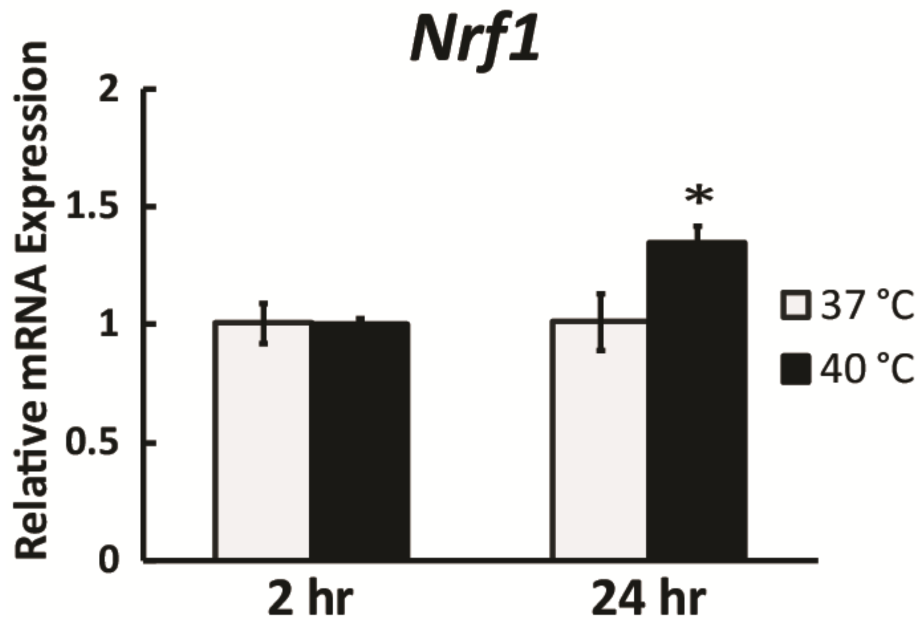


Figure 7A: Mild HS activates mitochondrial biogenesis program. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Nrf1* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. $^*P < 0.05$ compared to the control.

Figure 7B

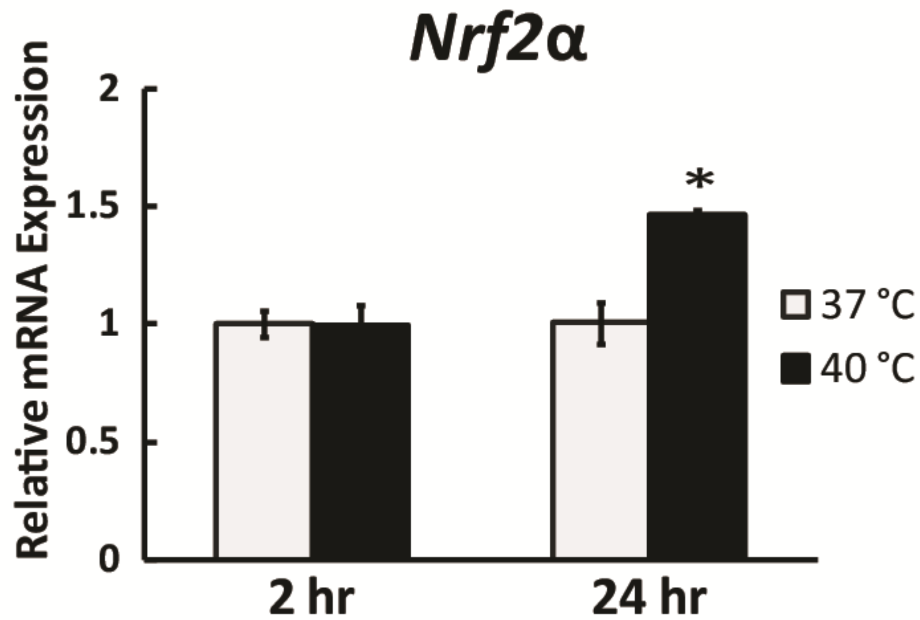


Figure 7B: Mild HS activates mitochondrial biogenesis program. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Nrf2α* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to the control.

Figure 7C

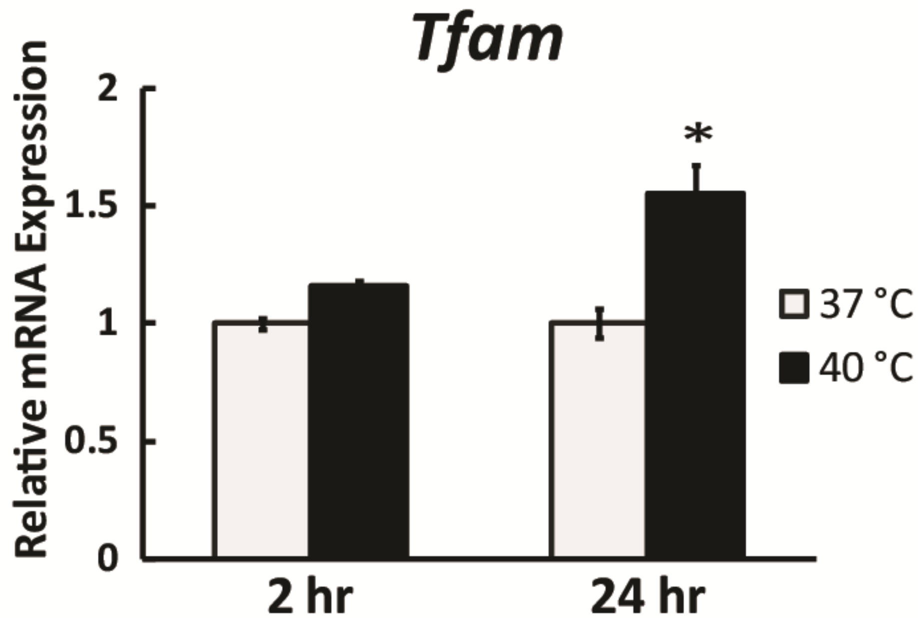


Figure 7C: Mild HS activates mitochondrial biogenesis program. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Tfam* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to the control.

Figure 7D

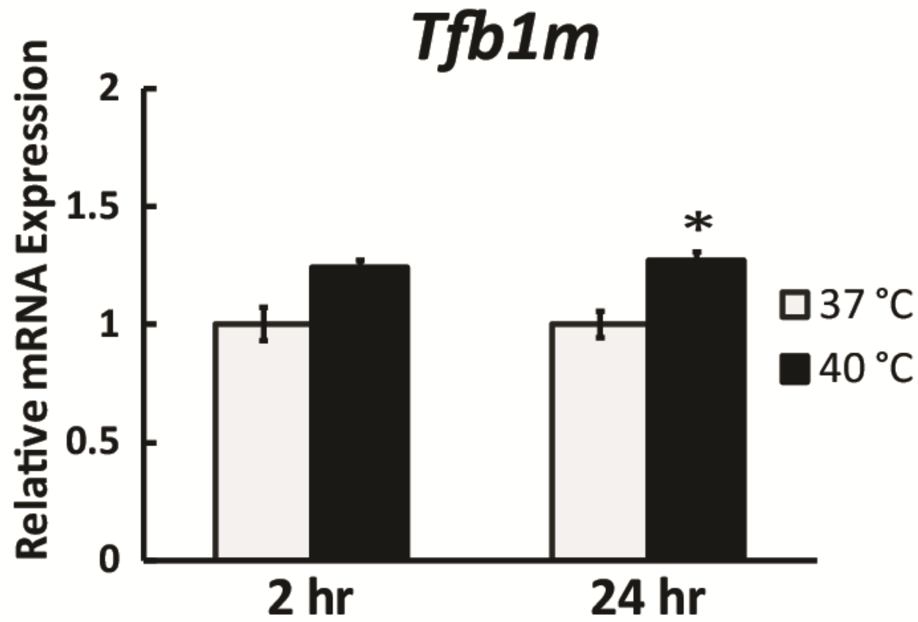


Figure 7D: Mild HS activates mitochondrial biogenesis program. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Tfb1m* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. **P* < 0.05 compared to the control.

Figure 7E

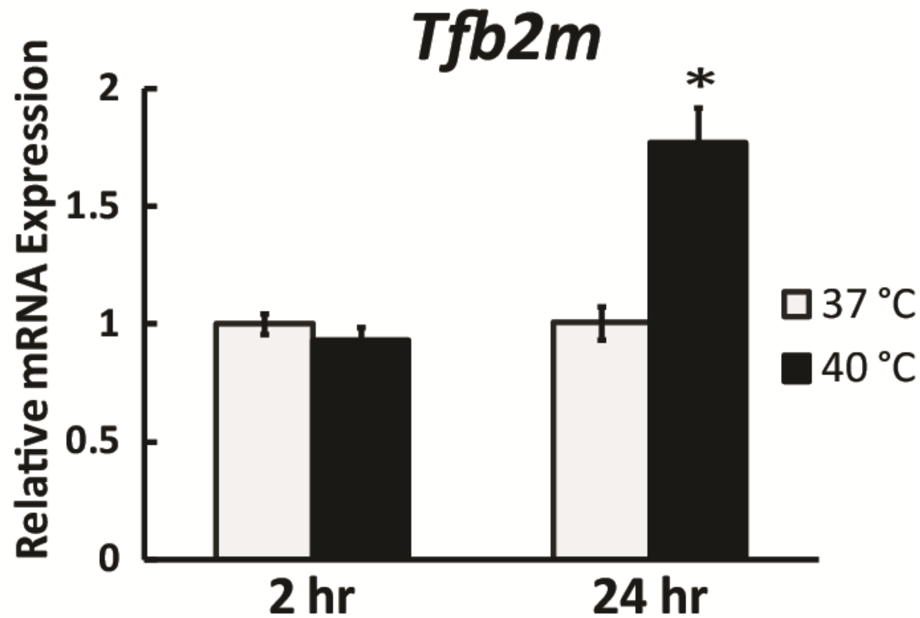


Figure 7E: Mild HS activates mitochondrial biogenesis program. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Tfb2m* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to the control.

D. Mild heat stress induces gene expression for mitochondrial components

Because activation of the mitochondrial biogenesis transcriptional cascade should lead to increased expression of mitochondrial components, we quantified the mRNA levels of mitochondrial oxidative proteins including cytochrome c (*Cycs*), nuclear-encoded cytochrome c oxidase subunit IV (*Cox4*) and mtDNA-encoded respiratory subunit *Cox2*. No significant changes in the mRNA levels of *Cycs*, *Cox2* and *Cox4* were observed 2 hr after mild HS while significant increases were seen in all of them 24 hr after treatment (1.40-fold, $P < 0.05$, 1.77-fold, $P < 0.05$ and 1.74-fold, $P < 0.05$ respectively) (Figures 8A-C). In summary, not only the regulatory genes, but also genes of mitochondrial components increased after mild HS.

In addition to the genes involved in mitochondrial biogenesis, we also measured the transcription of glucose transporter *Glut4*, another target regulated by PGC-1α. No significant change in the mRNA levels of *Glut4* was observed 2 hr after mild HS while an increase of 1.45-fold ($P < 0.05$) was seen after 24 hr (Figure 8D). At the same time, there was no difference observed in mRNA levels of lactate dehydrogenase isoform A (*Ldha*) after mild HS after 2 or 24 hr (data not shown).

Figure 8A

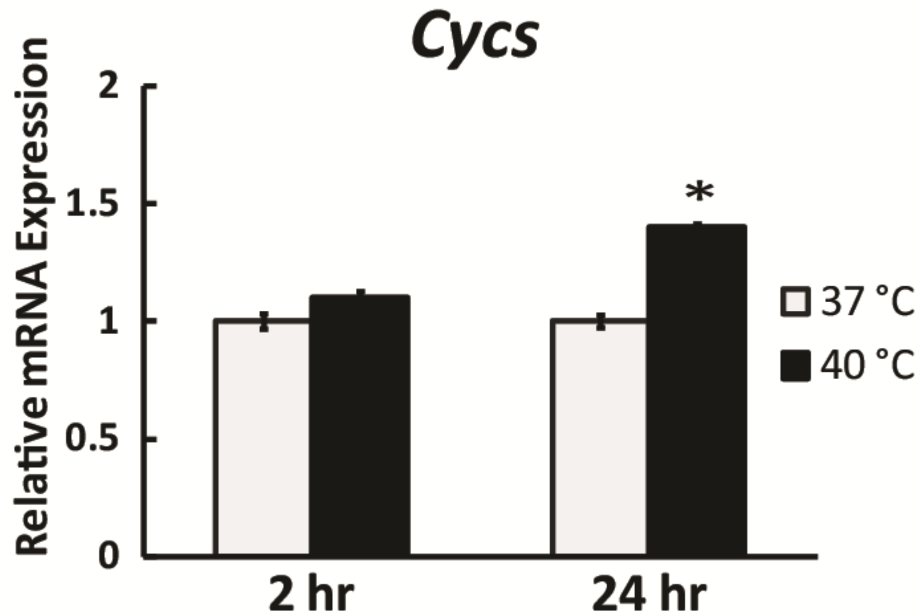


Figure 8A: Mild HS increases *Cycs* mRNA expression. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Cycs* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to the control.

Figure 8B

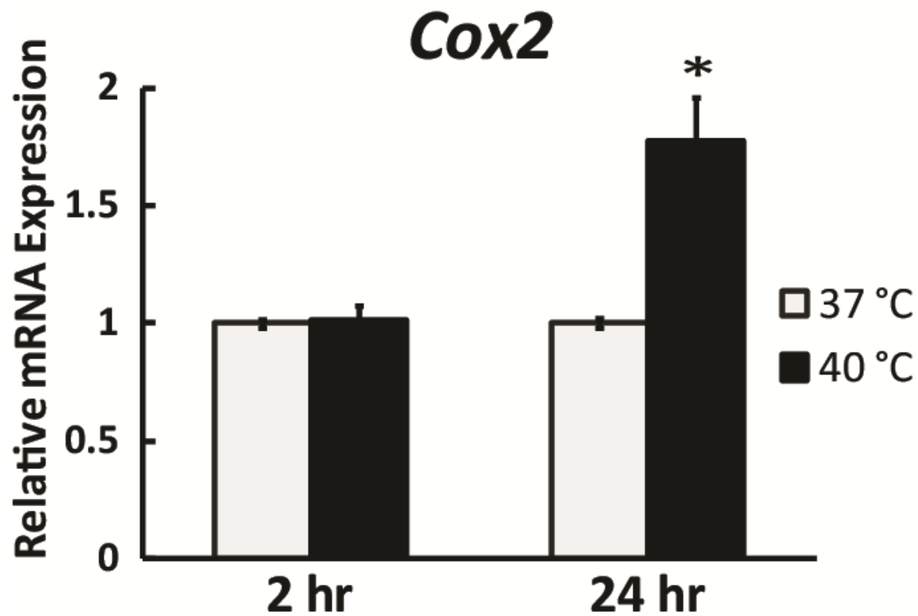


Figure 8B: Mild HS increases *Cox2* mRNA expression. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Cox2* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. **P* < 0.05 compared to the control.

Figure 8C

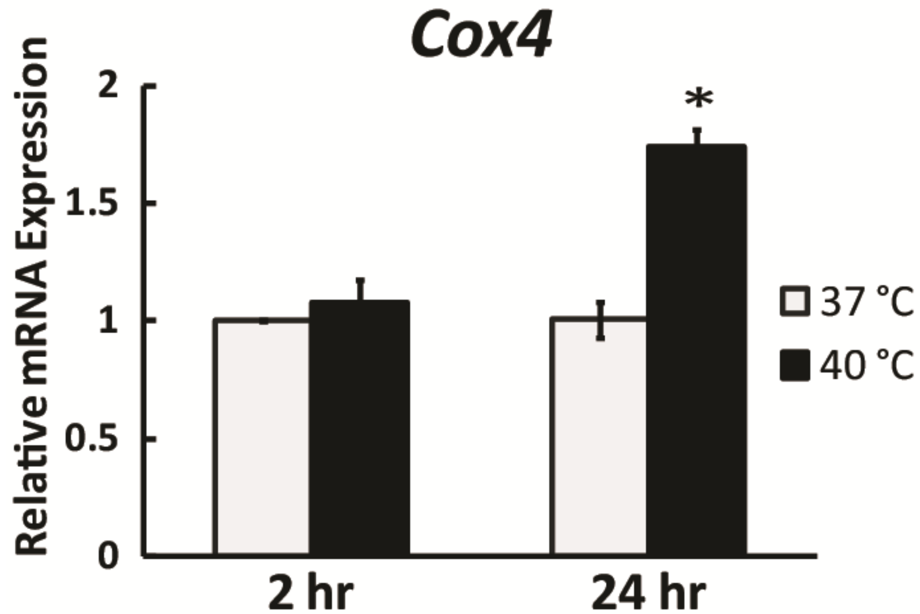


Figure 8C: Mild HS increases *Cox4* mRNA expression. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Cox4* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to the control.

Figure 8D

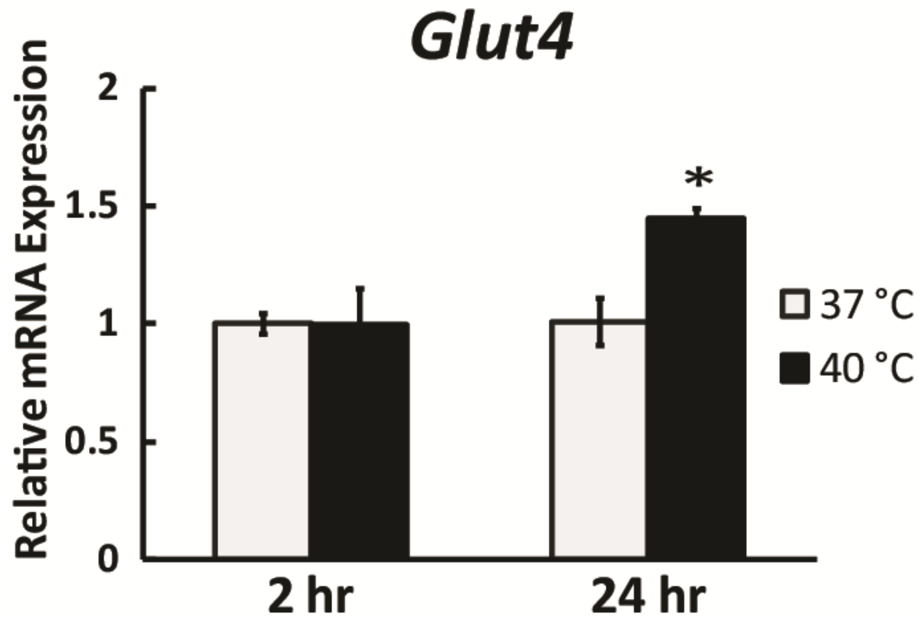


Figure 8D: Mild HS increases *Glut4* mRNA expression. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Glut4* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to the control.

E. Mild heat stress induces cytoprotective heat shock response

The heat-shock response is a conserved protective reaction of cells to elevated temperatures. The mRNA expression of stress-inducible *Hspa1a* (aka *Hsp72*) and *Hspa1b*, both members of the heat shock protein 70-kD family, increased by more than 100-fold when measured one hr after mild HS (Data not shown). Two hr and 24 hr after mild HS, the mRNA levels were still significantly higher for *Hspa1a* (3.41-fold, $P < 0.05$ and 1.82-fold $P < 0.05$) (Figure 9A) and for *Hspa1b* (4.98-fold, $P < 0.05$ and 1.67–fold, $P < 0.05$) (Figure 9B). A significant increase in HSP70 protein levels was also observed in dissected rat soleus muscles exposed to mild HS at 40°C for 1 hr and harvested immediately (Figure 9C). Our data show that mild HS within physiological range is sufficient to induce a cytoprotective heat shock response.

A variety of mitochondrial chaperones provide indispensable functions for mitochondrial biogenesis. The most abundant and important chaperone for mitochondrial function is mitochondrial heat shock protein 70 kDa (mtHsp70) which is involved in protein translocation into the mitochondria (70). Together with other inner membrane proteins of the mitochondria, membrane-associated mtHsp70 forms an import motor complex for polypeptide movement and unfolding during preprotein membrane translocation in an ATP-dependent manner. In addition, mtHsp70 in the matrix plays an essential role in the folding of newly imported proteins and in the biosynthesis of mitochondrially encoded proteins. Our data showed that mtHsp70 protein levels remained unchanged 24 hr after mild HS (Figure 9D).

Figure 9A

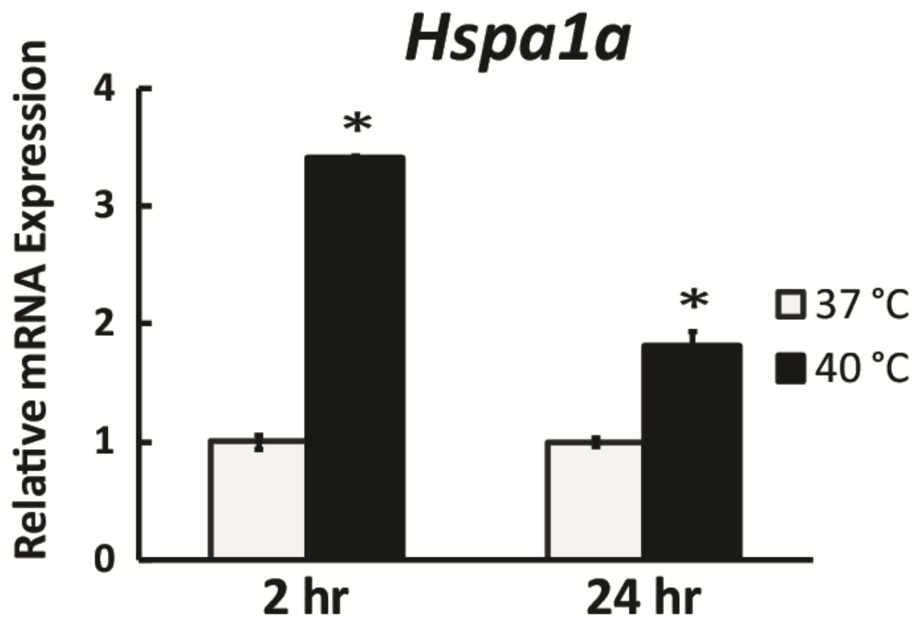


Figure 9A: Mild HS activates heat shock response. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Hspa1a* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to the control.

Figure 9B

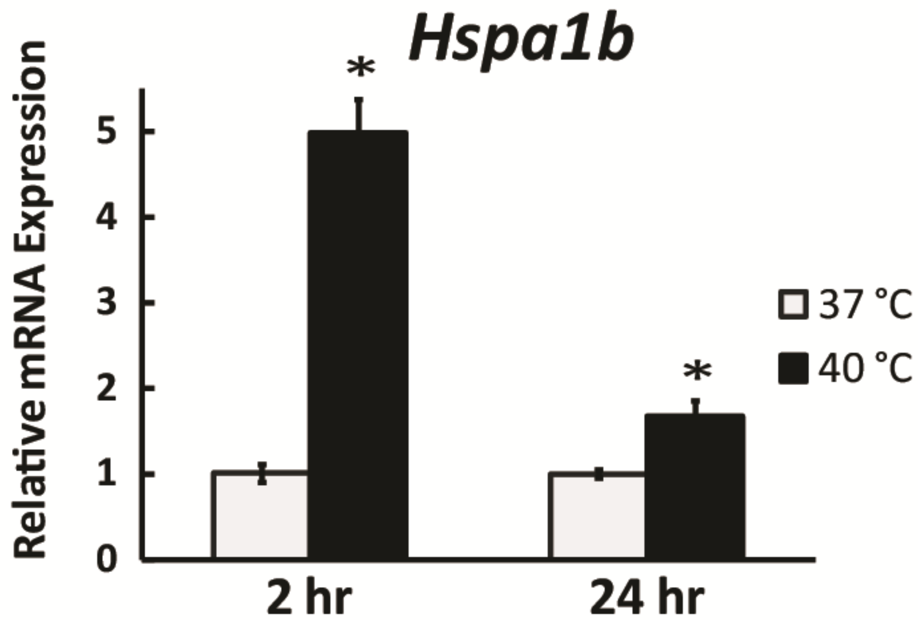


Figure 9B: Mild HS activates heat shock response. C2C12 myotubes were exposed to mild HS for one hr and then returned to the normal culture environment (37°C). Cells were harvested after 2- and 24-hr recovery periods. The mRNA levels of *Hspa1b* were determined by quantitative PCR and normalized to 18S ribosomal RNA. Results are expressed as means ± SEM of triplicate samples in one representative experiment from three independent trials. *$P < 0.05$ compared to the control.

Figure 9C

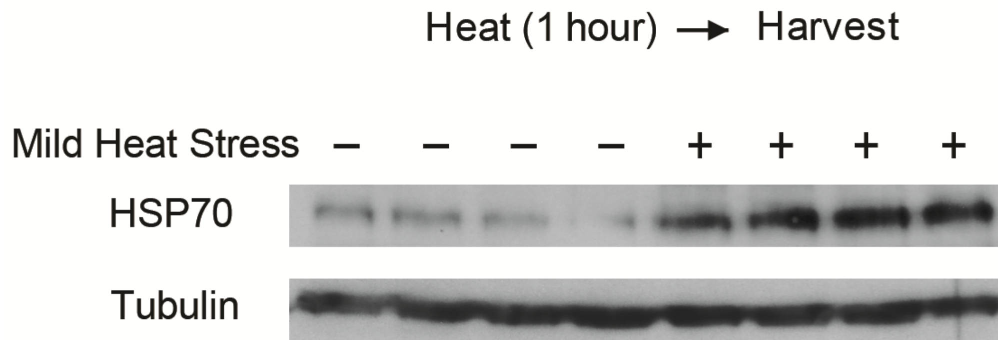
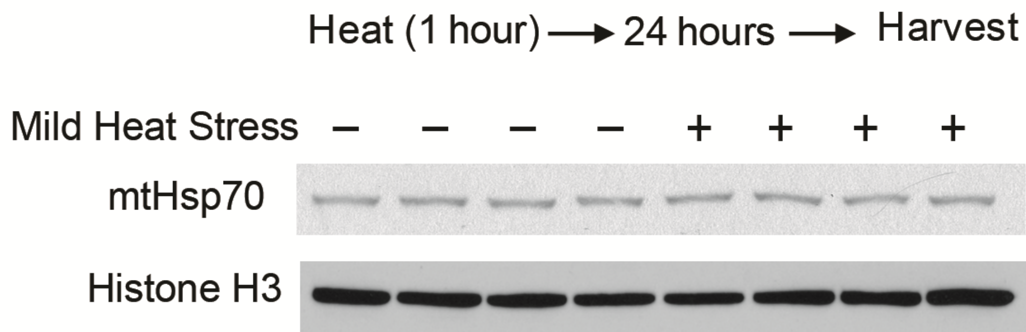


Figure 9C: Mild HS activates heat shock response in rat soleus muscle. Dissected Rat soleus muscles were exposed to mild HS at 40°C for 1 hr and harvested immediately for Western blot. Results were normalized to α-Tubulin.

Figure 9D



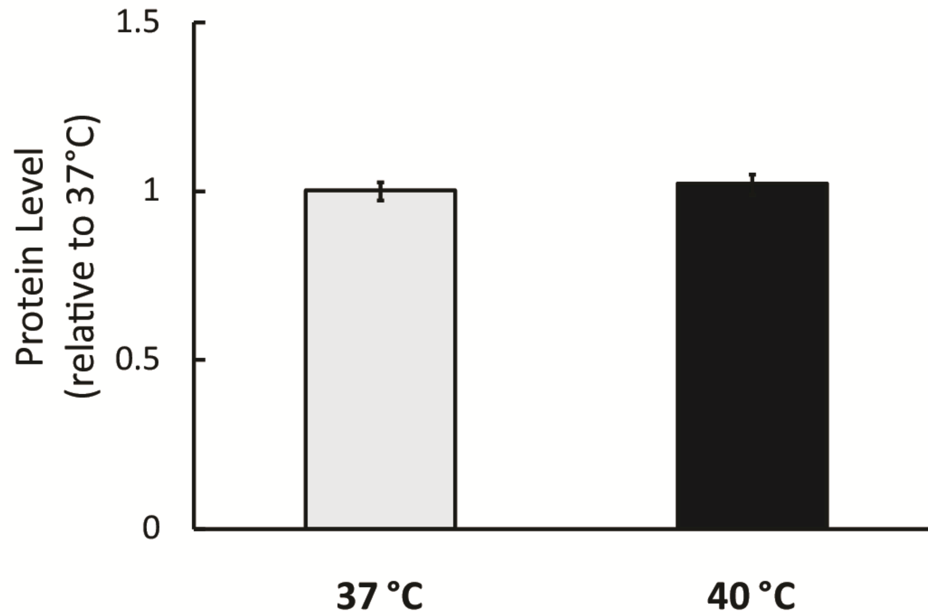


Figure 9D: mtHsp70 expression after Mild HS. C2C12 myotubes were exposed to 1-hr mild HS (40°C) and then returned to the normal culture environment (37°C). After a 24-hr recovery period, cells were harvested for Western blot. Results were normalized to Histone H3. Quantification of band intensity is shown below the Western blot. Results are given as the means ± SEM from four independent trials.* $P < 0.05$ compared to the control.

F. Repeated mild heat stress increases the expression of PGC-1α and oxidative phosphorylation subunit proteins as well as mitochondrial DNA copy number

Our initial studies demonstrated that a single bout of mild HS is capable of activating the expression of a diverse group of genes. To further characterize the adaptive response to mild HS in myotubes, repeated mild HS was used to mimic the repeated HS that occurs in muscle as the result of regular exercise.

C2C12 myotubes were exposed to one hr of mild HS per day for 5 days and then harvested for subsequent assays. With regard to 1-hr vs. 5-day comparisons we noted that the responses to treatments may not be proportional to the durations of treatment. For example, there was no significant difference in SIRT1 protein expression observed between control and HS groups (data not shown), therefore a bad example. The expression of important signaling transduction molecules tends to be transient. For example, AMPK kinase activities were halted immediately if cells in heat shock were returned to 37°C (16). Therefore, we focused on the regulatory molecules after one bout of mild HS and the protein and mitochondrial DNA end products after repeated heat treatments.

After a 5-day treatment a significant increase in PGC-1α protein expression was seen (1.58 fold, $P < 0.05$) (Figure 10A). Repeated mild HS significantly increased the protein levels of 4 representative oxidative phosphorylation subunits, Complex I subunit NDUFB8 (1.94 fold, $P < 0.05$), Complex II subunit 30 kDa (1.76 fold, $P < 0.05$), Complex III subunit Core 2 (1.78 fold, $P < 0.05$), and Complex V or ATP synthase subunit α (1.61 fold $P < 0.05$) (Figure 10A). The protein levels of Complex IV subunit I did not change significantly after treatment. In addition, the protein levels of major mitochondrial molecular chaperon mtHsp70 also significantly increased (1.26 fold $P < 0.05$) (Figure 10B).

Because mitochondria contain their own DNA encoding 13 proteins, measurements of mitochondria DNA copy number was used to verify changes in mitochondrial biogenesis levels (49). qPCR analysis showed that repeated mild HS for 5 days increased the mitochondrial DNA copy number by 1.68-fold ($P < 0.05$) (Figure 10C). Taken together, repeated mild HS for 5 days resulted in a significant increase in PGC-1α protein expression as well as in mitochondrial components including oxidative phosphorylation proteins and mitochondrial DNA.

Figure 10A

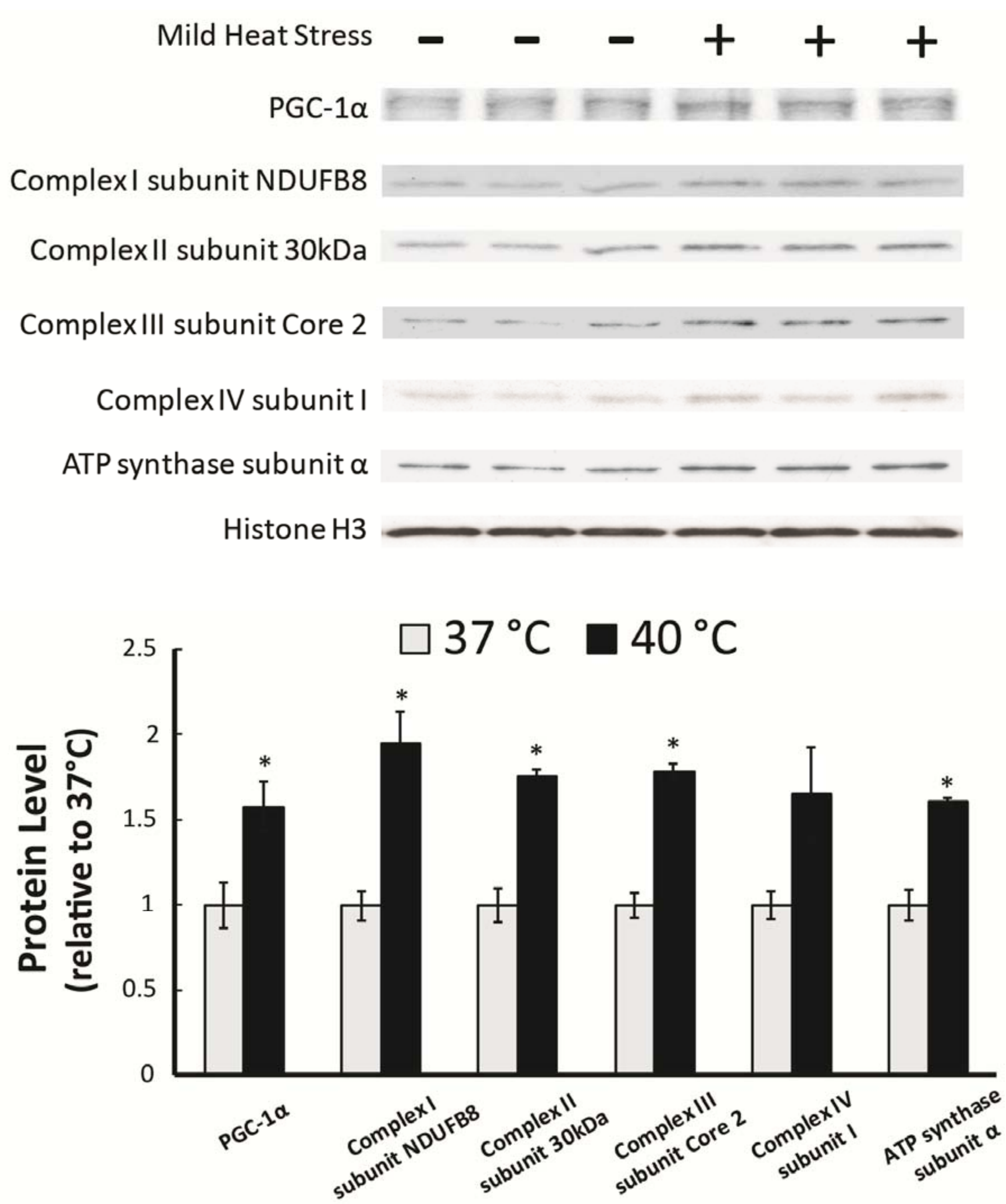


Figure 10A: Repeated mild HS increases the expression of PGC-1α and oxidative phosphorylation subunits. C2C12 myotubes were exposed to 1-hr HS for 5 days. 24 hr after the last treatment, cells were harvested and subjected to Western blot analysis using antibodies specific for PGC-1α and oxidative phosphorylation subunits. Protein levels were normalized to Histone H3.Quantification of band intensity is shown below the Western blot. Results are given as the means ± SEM from three independent trials.* $P < 0.05$ compared to control.

Figure 10B

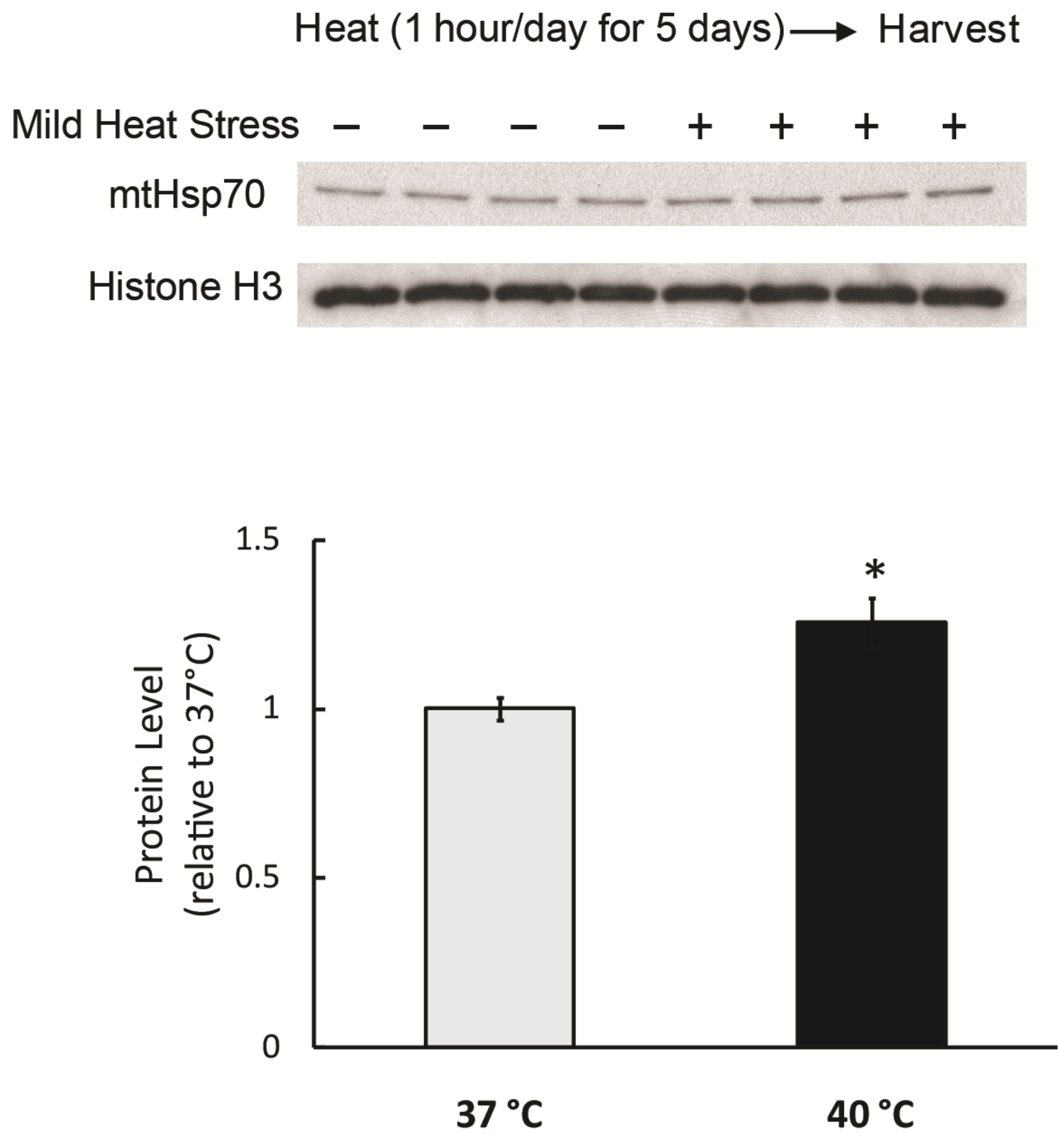


Figure 10B: Repeated mild HS increases the mtHsp70 expression. C2C12 myotubes were exposed to 1-hr HS for 5 days. 24 hr after the last treatment, cells were harvested and subjected to Western blot. Results were normalized to Histone H3. Quantification of band intensity is shown below the Western blot. Results are given as the means ± SEM from four independent trials.*$P < 0.05$ compared to the control.

Figure 10C

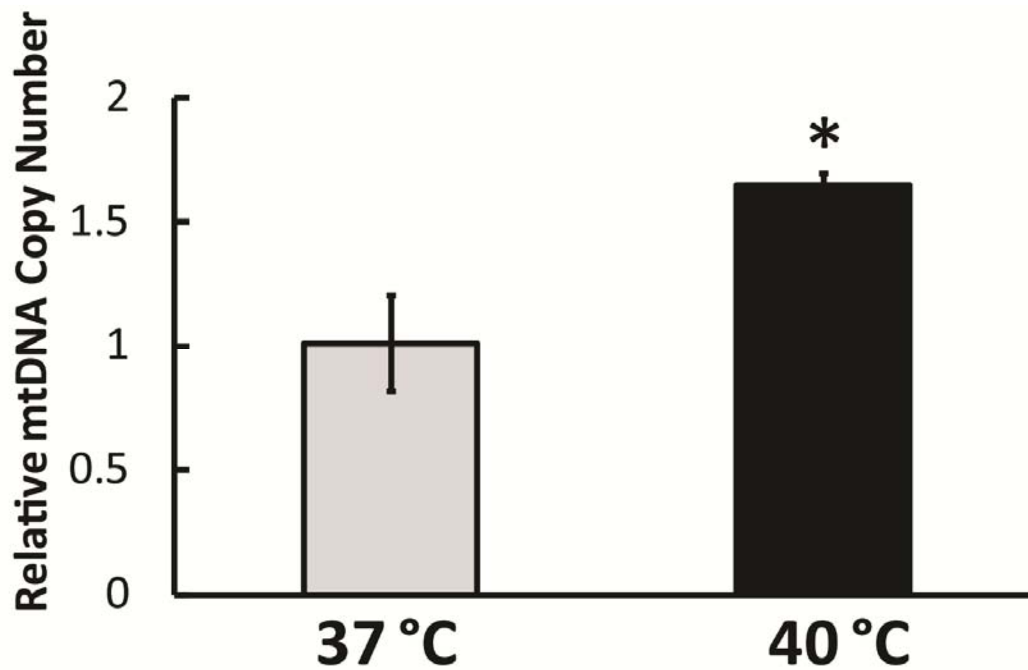


Figure 10C: Repeated mild HS increased mitochondrial DNA copy number. C2C12 myotubes were exposed to 1-hr mild HS for 5 days. 24 hr after the last treatment, cells were harvested for total DNA extraction. Using qPCR, the relative mitochondrial DNA copy number was determined by the ratio of mtDNA-encoded *Cox2* and nucleus-encoded 18S ribosomal RNA genes. Results are expressed as means ± SEM of triplicate samples in one representative experiment from six independent trials.*$P < 0.05$ compared to the control.

CHAPTER 4

DISCUSSION

This study demonstrates that mild HS is sufficient to induce mitochondrial biogenesis in C2C12 myotubes. Evidence that mild HS induces mitochondrial biogenesis has been shown in two ways. First, key molecules of the mitochondrial biogenesis pathway, including energy-sensing AMPK and SIRT1 as well as their downstream targets such as PGC-1α, are activated by mild HS. Second, levels of mitochondrial components, including several oxidative phosphorylation proteins and mitochondrial DNA, increase after mild HS.

Heat production is a byproduct of oxidative and mechanical coupling inefficiencies during physical activity, and dissipating excess heat is essential at the evolutionary, organismal and cellular levels. Skeletal muscle accounts for 30 to 40% of the total human body mass (74), and many people who perform regular endurance exercise also undergo regular whole-body mild HS, unlike their more sedentary counterparts. In this study, we demonstrated the impact of mild heat stress in energy metabolism by showing heat-induced mitochondrial biogenesis and its mechanisms at the cellular level.

A recent study has shown that non-damaging running exercise for 45 minutes increases muscle temperature from 36.2°C to 40°C (53). The authors also showed that the protein levels of HSP72 (HSPa1a) significantly increased 48 hr post-exercise in muscle. Interestingly, passive heating protocol using a tank containing water at 45 °C failed to induce a significant increase of major heat shock proteins in human skeletal muscle (54). This suggests that non-heat stress factors such as increased intracellular calcium may be involved in exercise-induced heat shock response. It is also possible that the superficial heat modality using in that study did not sufficiently heat deep muscles because of the thermal insulation by subcutaneous tissue and the efficient removal of heat by increased cutaneous blood flow.

Exercise-induced expression of HSPs in the heart is also one of the mechanisms responsible for the cardioprotective effects of exercise (24). Hspa1a (aka Hsp72) and Hspa1b (aka Hsp 70) are members of the heat shock protein 70-kD family. In our study, the mRNA expression of stress-inducible Hspa1a increased by more than 1000-fold one hr after mild HS (data not shown). This dramatic change may be unrealistic because of little or no basal expression of this protein (19). Hspa1b is the major heat-inducible member with some basal expression. In our study Hspa1b increased by 415-fold when measured one hr after mild HS (Data not shown). Two hr after mild HS the relative mRNA levels of Hspa1b (4.98-fold, $P < 0.05$) was higher than that of Hspa1a (3.41-fold, $P < 0.05$), which may reflect Hspa1b's role as the major heat-inducible member (19). In summary, temperature increase within the physiological range is sufficient to induce the cytoprotective heat shock response.

In addition to the heat shock response, mild HS also activates energy sensing molecules

AMPK and SIRT1, which respond to various stresses by adjusting cellular metabolic activities. AMPK is a key cellular metabolic sensor and plays a role in regulation of whole body energy homeostasis (30). Our results showed that the activation of AMPK was immediately seen after mild HS (Figure 5A), which is consistent with the results of a previous investigation (16). A recent study showed that increase of Nampt expression during glucose restriction in C2C12 myoblasts was mediated by AMPK and implicated AMPK, Nampt and SIRT1 as components in a skeletal muscle nutrient-sensing response pathway (20). Another study showed that skeletal muscle Nampt increased in response to exercise in humans (17). We found that a single bout of mild HS is sufficient to increase Nampt levels. Combined with prior reports, this suggests that Nampt-mediated NAD biosynthesis may play a role in the regulation of AMPK-SIRT1-PGC-1α pathway and also correlate with the heat-induced mitochondrial biogenesis.

SIRT1 is well-known for its role in modulating the lifespan of a number of organisms including yeast, worms, flies and mice with restricted caloric intake (45). Similarly, mild HS has also long been reported to extend lifespan in different species (42, 48, 66), though the mechanism is not yet clear. Recently, Westerheide *et al*. showed that SIRT1 directly deacetylates heat shock factor 1 and thereby regulates the acute heat shock response in mammalian cells (72). That result may mean that SIRT1 plays a role in heat-induced life span extension. Our results showed that SIRT1 protein levels significantly increased 24 hr after mild HS. To our knowledge, this is the first report showing the direct upregulation of SIRT1 protein expression by heat. This may mean that SIRT1 not only functions in the early stages of the heat shock response, but may also provide important long-term functions after HS is removed. Accordingly, upregulation of SIRT1 may play a crucial role in two renowned environmental stressors that extend lifespan in animals: calorie restriction and mild heat stress.

Based on the evidence of the increase of PGC-1α promoter activity and mRNA levels, our data show that PGC-1α is transcriptionally modulated by mild HS. Interestingly, levels of PGC-1α were initially found to be greatly induced in muscle and brown fat by cold exposure (58). The common effect of cold exposure and excess heat on cells is an increase in cellular energy requirement. Taken together with results of previous studies, there is clear evidence that PGC-1α plays a role in response to temperature fluctuations in cells.

Glucose uptake is a rate-limiting step in muscle glucose metabolism (71), and both gain-of-function and loss-of-function mutations have shown that GLUT4 is essential in the maintenance of normal glucose homeostasis (10, 78). As well, in skeletal muscle, *Glut4* is one of the targets regulated by PGC-1α (50). In our studies, *Glut4* transcription was

significantly increased by mild HS, possibly due to an increase of PGG-1α expression. Thus, results of our study may reveal an additional factor that, among others (15, 26), contributes to the heat-induced improvement of glucose tolerance and insulin sensitivity.

Multiple lines of evidence have shown that endurance exercise training results in an increase in skeletal muscle mitochondrial biogenesis (34). The physiological significance of mitochondrial biogenesis is the enhancement of cellular oxidative phosphorylation, which improves endurance performance at the organismal level. We found that the levels of several oxidative phosphorylation proteins and mitochondrial DNA copy number significantly increased after repeated mild HS. Because we show that in C2C12 myotubes mild HS increases markers of mitochondrial biogenesis similar to what occurs in regular endurance training, it is possible that muscle heat production during exercise plays a role in mitochondrial biogenesis. One limitation of this report is that C2C12 myotube culture system may not entirely reflect what occurs in vivo. Another limitation is that the data presented here do not establish a causal link between AMPK-SIRT1-PGC1α activation and mitochondrial biogenesis. Still, on the basis of our findings it is likely that, by itself, heat stress both affects mitochondrial biogenesis and increases the gain in response to other, contraction-induced, activators of mitochondrial biogenesis.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

A. Conclusion

Mitochondrial biogenesis is a vital and exciting area of cell biology. Lack of normal mitochondrial function has a negative impact in insulin resistance and aging, among others (57). In this report, we show that mild HS is sufficient to induce mitochondrial biogenesis associated with activation of the AMPK-SIRT1-PGC-1α pathway in C2C12 myotubes. The proposed model is illustrated in Figure 11. The results presented here suggest the possibility of developing treatments for conditions caused by mitochondrial deficit using heat modalities when the ability to exercise is impaired by orthopedic or other conditions that limit mobility.

B. Future Directions

1. To establish a cause-effect relationship between the AMPK-SIRT1-PGC-1α pathway and heat-induced mitochondrial biogenesis, it is necessary to systemically use agonists or gain-of-function approaches to establish sufficiency and inhibitors or loss-of-function approaches to establish necessity for key proteins in this pathway.

2. To further characterize the role of PGC-1α in heat-induced mitochondrial biogenesis, it is necessary to use an overexpression system to investigate posttranslational modifications (i.e. phosphorylation and acetylation) of PGC-1α.

3. The key experiments should be repeated using primary muscle cell cultures.

4. The experiments need to be repeated in animal models to test whether the temperature-induced effects are reproducible in vivo.

5. In addition to myotubes/primary muscle cell cultures, experiments of mild HS can be performed in cells from different tissues such as hepatocytes or adipocytes to investigate the potentially beneficial effects on health.

6. It is necessary to investigate the role of Sir2 activity and expression in the mild HS-induced longevity in yeast, worms, and flies.

7. To investigate possible practical clinical applications, it may be worthwhile to conduct experiments applying mild heat on specific internal organs using deep heat modalities such as shortwave or applying whole body mild heat stress using extracorporeal circulation in experimental animal models.

8. Because hemodialysis requires access to the circulatory system, it may offer potential therapeutic value to study the effect of higher dialysate temperature on the metabolic profile and insulin sensitivity in selective patients receiving regular hemodialysis treatment.

Figure 11

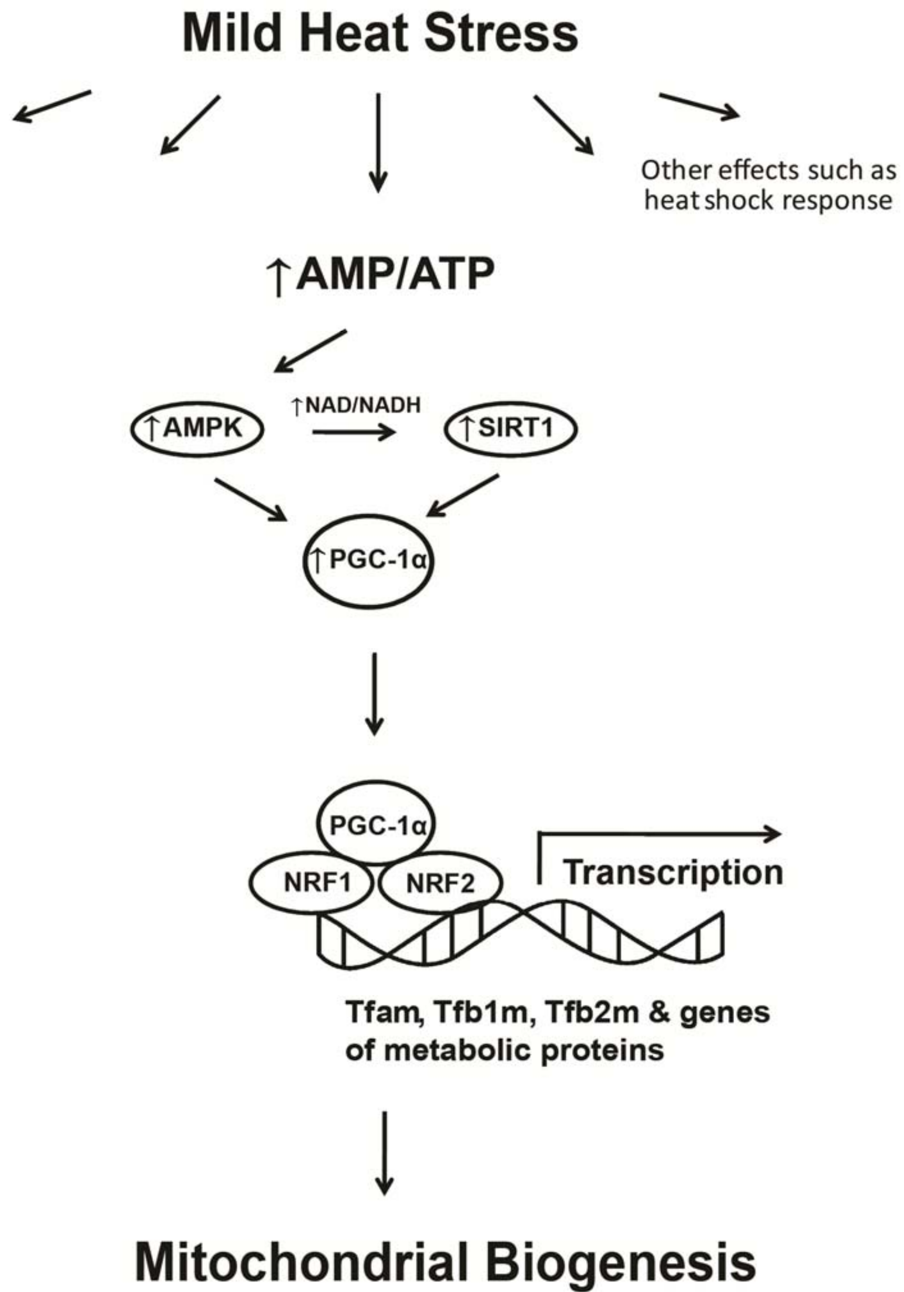


Figure 11: Proposed model of mild HS-induced mitochondrial biogenesis correlating with activation of the AMPK-SIRT1- PGC-1α pathway. When cells are exposed to mild HS, AMPK activity rapidly increases. AMPK then upregulates SIRT1 expression by increasing the cellular NAD+/NADH ratio. Together, AMPK and SIRT1 enhance the expression of the downstream molecule PGC-1α. PGC-1α further co-activates NRF1 and NRF2 to promote the expression of key components of the mitochondrial transcription and translation machinery for mitochondrial biogenesis.

REFERENCES

1. **Alberts B, Johnson A, Lewis J, Raff M, Roberts K, and Walter P.** *Molecular biology of the cell*. New York: Garland Science, 2002.
2. **Amat R, Planavila A, Chen SL, Iglesias R, Giralt M, and Villarroya F.** SIRT1 controls the transcription of the peroxisome proliferator-activated receptor-gamma Co-activator-1alpha (PGC-1alpha) gene in skeletal muscle through the PGC-1alpha autoregulatory loop and interaction with MyoD. *J Biol Chem* 284: 21872-21880, 2009.
3. **Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, and Holloszy JO.** Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J* 16: 1879-1886, 2002.
4. **Bai RK, Perng CL, Hsu CH, and Wong LJ.** Quantitative PCR analysis of mitochondrial DNA content in patients with mitochondrial disease. *Ann N Y Acad Sci* 1011: 304-309, 2004.
5. **Blander G and Guarente L.** The Sir2 family of protein deacetylases. *Annu Rev Biochem* 73: 417-435, 2004.
6. **Bramble DM and Lieberman DE.** Endurance running and the evolution of Homo. *Nature* 432: 345-352, 2004.
7. **Brooks GA, Fahey TD, White TP, and Baldwin KM.** *Exercise physiology: human bioenergetics and its applications*: McGraw-Hill New York, 2005.
8. **Brooks GA, Hittelman KJ, Faulkner JA, and Beyer RE.** Temperature, skeletal muscle mitochondrial functions, and oxygen debt. *Am J Physiol* 220: 1053-1059, 1971.
9. **Brooks GA, Hittelman KJ, Faulkner JA, and Beyer RE.** Tissue temperatures and whole-animal oxygen consumption after exercise. *Am J Physiol* 221: 427-431, 1971.
10. **Brozinick JT, Jr., McCoid SC, Reynolds TH, Nardone NA, Hargrove DM, Stevenson RW, Cushman SW, and Gibbs EM.** GLUT4 overexpression in db/db mice dose-dependently ameliorates diabetes but is not a lifelong cure. *Diabetes* 50: 593-600, 2001.
11. **Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, and Auwerx J.** AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* 458: 1056-1060, 2009.
12. **Canto C, Jiang LQ, Deshmukh AS, Mataki C, Coste A, Lagouge M, Zierath JR, and Auwerx J.** Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle. *Cell Metab* 11: 213-219, 2010.
13. **Carrier DR, Kapoor AK, Kimura T, Nickels MK, Satwanti, Scott EC, So JK, and Trinkaus E.** The Energetic Paradox of Human Running and Hominid Evolution [and Comments and Reply]. *Current Anthropology* 25: 483-495, 1984.
14. **Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R, and Williams RS.** A calcineurin-dependent transcriptional pathway

controls skeletal muscle fiber type. *Genes Dev* 12: 2499-2509, 1998.

15. **Chung J, Nguyen AK, Henstridge DC, Holmes AG, Chan MH, Mesa JL, Lancaster GI, Southgate RJ, Bruce CR, Duffy SJ, Horvath I, Mestril R, Watt MJ, Hooper PL, Kingwell BA, Vigh L, Hevener A, and Febbraio MA.** HSP72 protects against obesity-induced insulin resistance. *Proc Natl Acad Sci U S A* 105: 1739-1744, 2008.

16. **Corton JM, Gillespie JG, and Hardie DG.** Role of the AMP-activated protein kinase in the cellular stress response. *Curr Biol* 4: 315-324, 1994.

17. **Costford SR, Bajpeyi S, Pasarica M, Albarado DC, Thomas SC, Xie H, Church TS, Jubrias SA, Conley KE, and Smith SR.** Skeletal muscle NAMPT is induced by exercise in humans. *Am J Physiol Endocrinol Metab* 298: E117-126, 2010.

18. **Falkenberg M, Larsson NG, and Gustafsson CM.** DNA replication and transcription in mammalian mitochondria. *Annu Rev Biochem* 76: 679-699, 2007.

19. **Feige U and Polla BS.** Hsp70--a multi-gene, multi-structure, multi-function family with potential clinical applications. *Experientia* 50: 979-986, 1994.

20. **Fulco M, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve AA, and Sartorelli V.** Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. *Dev Cell* 14: 661-673, 2008.

21. **Gaesser GA and Brooks GA.** Muscular efficiency during steady-rate exercise: effects of speed and work rate. *J Appl Physiol* 38: 1132-1139, 1975.

22. **Garber CE, Blissmer B, Deschenes MR, Franklin BA, Lamonte MJ, Lee IM, Nieman DC, and Swain DP.** Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise. *Med Sci Sports Exerc* 43: 1334-1359, 2011.

23. **Gleyzer N, Vercauteren K, and Scarpulla RC.** Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. *Mol Cell Biol* 25: 1354-1366, 2005.

24. **Golbidi S and Laher I.** Molecular mechanisms in exercise-induced cardioprotection. *Cardiol Res Pract* 2011: 972807, 2011.

25. **Goto M, Terada S, Kato M, Katoh M, Yokozeki T, Tabata I, and Shimokawa T.** cDNA Cloning and mRNA analysis of PGC-1 in epitrochlearis muscle in swimming-exercised rats. *Biochem Biophys Res Commun* 274: 350-354, 2000.

26. **Gupte AA, Bomhoff GL, Swerdlow RH, and Geiger PC.** Heat treatment improves glucose tolerance and prevents skeletal muscle insulin resistance in rats fed a high-fat diet. *Diabetes* 58: 567-578, 2009.

27. **Handschin C, Rhee J, Lin J, Tarr PT, and Spiegelman BM.** An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. *Proc Natl Acad Sci U S A* 100: 7111-7116, 2003.

28. **Handschin C and Spiegelman BM.** Peroxisome proliferator-activated receptor gamma

coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocr Rev* 27: 728-735, 2006.

29. **Hardie DG.** AMPK: a key regulator of energy balance in the single cell and the whole organism. *Int J Obes (Lond)* 32 Suppl 4: S7-12, 2008.

30. **Hardie DG and Sakamoto K.** AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology (Bethesda)* 21: 48-60, 2006.

31. **He ZH, Bottinelli R, Pellegrino MA, Ferenczi MA, and Reggiani C.** ATP consumption and efficiency of human single muscle fibers with different myosin isoform composition. *Biophys J* 79: 945-961, 2000.

32. **Hendrick JP and Hartl FU.** Molecular chaperone functions of heat-shock proteins. *Annu Rev Biochem* 62: 349-384, 1993.

33. **Hochachka PW and Somero GN.** *Biochemical adaptation: mechanism and process in physiological evolution*: Oxford University Press, USA, 2002.

34. **Holloszy JO and Coyle EF.** Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *J Appl Physiol* 56: 831-838, 1984.

35. **Hooper PL.** Hot-tub therapy for type 2 diabetes mellitus. *N Engl J Med* 341: 924-925, 1999.

36. **Howe AS and Boden BP.** Heat-related illness in athletes. *Am J Sports Med* 35: 1384-1395, 2007.

37. **Imai S.** The NAD World: a new systemic regulatory network for metabolism and aging--Sirt1, systemic NAD biosynthesis, and their importance. *Cell Biochem Biophys* 53: 65-74, 2009.

38. **Imai S, Armstrong CM, Kaeberlein M, and Guarente L.** Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 403: 795-800, 2000.

39. **Jager S, Handschin C, St-Pierre J, and Spiegelman BM.** AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proc Natl Acad Sci U S A* 104: 12017-12022, 2007.

40. **Joseph AM, Pilegaard H, Litvintsev A, Leick L, and Hood DA.** Control of gene expression and mitochondrial biogenesis in the muscular adaptation to endurance exercise. *Essays Biochem* 42: 13-29, 2006.

41. **Jubrias SA, Vollestad NK, Gronka RK, and Kushmerick MJ.** Contraction coupling efficiency of human first dorsal interosseous muscle. *J Physiol* 586: 1993-2002, 2008.

42. **Khazaeli AA, Tatar M, Pletcher SD, and Curtsinger JW.** Heat-induced longevity extension in Drosophila. I. Heat treatment, mortality, and thermotolerance. *J Gerontol A Biol Sci Med Sci* 52: B48-52, 1997.

43. **Kirkwood SP, Munn EA, and Brooks GA.** Mitochondrial reticulum in limb skeletal muscle. *Am J Physiol* 251: C395-402, 1986.

44. **Koulmann N and Bigard AX.** Interaction between signalling pathways involved in

skeletal muscle responses to endurance exercise. *Pflugers Arch* 452: 125-139, 2006.

45. **Kwon HS and Ott M.** The ups and downs of SIRT1. *Trends Biochem Sci* 33: 517-525, 2008.

46. **Lim CL, Byrne C, and Lee JK.** Human thermoregulation and measurement of body temperature in exercise and clinical settings. *Ann Acad Med Singapore* 37: 347-353, 2008.

47.**Lindquist S and Craig EA.** The heat-shock proteins. *Annu Rev Genet* 22: 631-677, 1988.

48. **Lithgow GJ, White TM, Melov S, and Johnson TE.** Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. *Proc Natl Acad Sci U S A* 92: 7540-7544, 1995.

49. **Medeiros DM.** Assessing mitochondria biogenesis. *Methods* 46: 288-294, 2008.

50. **Michael LF, Wu Z, Cheatham RB, Puigserver P, Adelmant G, Lehman JJ, Kelly DP, and Spiegelman BM.** Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci U S A* 98: 3820-3825, 2001.

51. **Molles MC.** *Ecology: concepts and applications*: McGraw-Hill Companies, 2009.

52.**Mootha VK, Handschin C, Arlow D, Xie X, St Pierre J, Sihag S, Yang W, Altshuler D, Puigserver P, Patterson N, Willy PJ, Schulman IG, Heyman RA, Lander ES, and Spiegelman BM.** Erralpha and Gabpa/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. *Proc Natl Acad Sci U S A* 101: 6570-6575, 2004.

53. **Morton JP, MacLaren DP, Cable NT, Bongers T, Griffiths RD, Campbell IT, Evans L, Kayani A, McArdle A, and Drust B.** Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise. *J Appl Physiol* 101: 176-182, 2006.

54.**Morton JP, Maclaren DP, Cable NT, Campbell IT, Evans L, Bongers T, Griffiths RD, Kayani AC, McArdle A, and Drust B.** Elevated core and muscle temperature to levels comparable to exercise do not increase heat shock protein content of skeletal muscle of physically active men. *Acta Physiol (Oxf)* 190: 319-327, 2007.

55. **Moyes CD and Schulte PM.** *Principles of animal physiology*: Pearson, 2006.

56. **Nelson FE, Ortega JD, Jubrias SA, Conley KE, and Kushmerick MJ.** High efficiency in human muscle: an anomaly and an opportunity? *J Exp Biol* 214: 2649-2653, 2011.

57. **Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, and Shulman GI.** Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* 300: 1140-1142, 2003.

58. **Puigserver P, Wu Z, Park CW, Graves R, Wright M, and Spiegelman BM.** A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92: 829-839, 1998.

59. **Revollo JR, Grimm AA, and Imai S.** The regulation of nicotinamide adenine

dinucleotide biosynthesis by Nampt/PBEF/visfatin in mammals. *Curr Opin Gastroenterol* 23: 164-170, 2007.

60. **Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, and Puigserver P.** Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* 434: 113-118, 2005.

61. **Ruderman NB, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F, and Ido Y.** AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab* 298: E751-760, 2010.

62. **Saltin B, Gagge AP, Bergh U, and Stolwijk JA.** Body temperatures and sweating during exhaustive exercise. *J Appl Physiol* 32: 635-643, 1972.

63. **Saltin B, Gagge AP, and Stolwijk JA.** Muscle temperature during submaximal exercise in man. *J Appl Physiol* 25: 679-688, 1968.

64. **Saraste M.** Oxidative phosphorylation at the fin de siecle. *Science* 283: 1488-1493, 1999.

65. **Schwer B and Verdin E.** Conserved metabolic regulatory functions of sirtuins. *Cell Metab* 7: 104-112, 2008.

66. **Shama S, Lai CY, Antoniazzi JM, Jiang JC, and Jazwinski SM.** Heat stress-induced life span extension in yeast. *Exp Cell Res* 245: 379-388, 1998.

67. **Smith NP, Barclay CJ, and Loiselle DS.** The efficiency of muscle contraction. *Prog Biophys Mol Biol* 88: 1-58, 2005.

68. **Taylor EB, Lamb JD, Hurst RW, Chesser DG, Ellingson WJ, Greenwood LJ, Porter BB, Herway ST, and Winder WW.** Endurance training increases skeletal muscle LKB1 and PGC-1alpha protein abundance: effects of time and intensity. *Am J Physiol Endocrinol Metab* 289: E960-968, 2005.

69. **Terada S, Kawanaka K, Goto M, Shimokawa T, and Tabata I.** Effects of high-intensity intermittent swimming on PGC-1alpha protein expression in rat skeletal muscle. *Acta Physiol Scand* 184: 59-65, 2005.

70. **Voos W and Rottgers K.** Molecular chaperones as essential mediators of mitochondrial biogenesis. *Biochim Biophys Acta* 1592: 51-62, 2002.

71. **Wallberg-Henriksson H and Zierath JR.** GLUT4: a key player regulating glucose homeostasis? Insights from transgenic and knockout mice (review). *Mol Membr Biol* 18: 205-211, 2001.

72. **Westerheide SD, Anckar J, Stevens SM, Jr., Sistonen L, and Morimoto RI.** Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. *Science* 323: 1063-1066, 2009.

73.**Whipp BJ and Wasserman K.** Efficiency of muscular work. *J Appl Physiol* 26: 644-648, 1969.

74. **Woodard HQ and White DR.** The composition of body tissues. *Br J Radiol* 59: 1209-1218, 1986.

75. **Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, and Spiegelman BM.** Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98: 115-124, 1999.
76. **Yang Z, Kahn BB, Shi H, and Xue BZ.** Macrophage alpha1 AMP-activated protein kinase (alpha1AMPK) antagonizes fatty acid-induced inflammation through SIRT1. *J Biol Chem* 285: 19051-19059, 2010.
77. **Yoon JC, Ng A, Kim BH, Bianco A, Xavier RJ, and Elledge SJ.** Wnt signaling regulates mitochondrial physiology and insulin sensitivity. *Genes Dev* 24: 1507-1518, 2010.
78. **Zisman A, Peroni OD, Abel ED, Michael MD, Mauvais-Jarvis F, Lowell BB, Wojtaszewski JF, Hirshman MF, Virkamaki A, Goodyear LJ, Kahn CR, and Kahn BB.** Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med* 6: 924-928, 2000.