Effects of core temperature on exercise-induced mitochondrial biogenesis: Potential role of PGC-1α, AMPK eNOS, SIRT1, and SIRT3

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Effects of core temperature on exercise-induced mitochondrial biogenesis: Potential role of PGC-1α, AMPK eNOS, SIRT1, and SIRT3

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelors of Science in Kinesiology and Health Sciences from The College of William and Mary

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Abstract

Previously, we have demonstrated a dramatic increase in mitochondrial biogenesis in skeletal muscle of rats exercise trained while maintaining a constant core temperature. In this study, we explored the potential mechanisms of increased mitochondrial biogenesis in this model by examining the expression of PGC-1α, AMPK, eNOS, SIRT1 and SIRT3. Female, Sprague-Dawley rats (5 mos of age) were divided into three groups sedentary (S), exercise in 22°C room (ET), and exercise while maintaining core temperature (E). Exercised animals trained for 5 weeks on a motor-driven treadmill at 30 m/min, 60 min/day, and 5 days/wk during the final 2 weeks. Core temperature was held constant in E by reducing room temperature to 6-8°C. Mitochondrial biogenesis was increased in cold-trained animals versus room temperature-trained animals as indicated by a significant (P<0.05) increase in cytochrome oxidase activity in the cold training group. Hsp70 expression was significantly (P< 0.05) increased in both ET and E rats versus SED, but ET was also significantly (P< 0.05) higher than the E group. Cold training was also less stressful than regular exercise; serum LDH was significantly (P< 0.05) elevated among ET tissues versus E tissues. There was no difference in PGC-1α, AMPK, or eNOS expression between any of the groups. SIRT1 was significantly (P< 0.05) decreased in rats trained at room temperature (ET), but unchanged in cold-trained rats (E). SIRT3 expression was significantly (P< 0.05) increased in rats trained at both room temperature and between 6-8°C, but no difference was observed between the two. The results of this study indicate that the effect of core temperature alters the pathway by which mitochondrial biogenesis occurs.
Background

Commonly referred to as the “powerhouses” of the cell, the mitochondria are the body’s primary producers of ATP, the chemical substrate used for energy. According to the United Mitochondrial Disease Foundation, the mitochondria are “responsible for creating more than 90% of the energy needed to sustain life and support growth” (UMDF, n.d.). However, energy production is not the only important role of the mitochondrion, it also plays a key role in proper development, apoptosis, and aging (Fernández-Moreno, Bornstein, Petit, & Garesse, 2000). Number and size of mitochondria are correlated with mitochondrial oxidative capacity (Kim, Wei, & Sowers, 2008). For example, muscle types that are continuously in use, such as the heart, have both higher mitochondrial activity and content than lesser-used muscles of the body (Jornayvaz & Shulman, 2010). Mitochondria are largely ubiquitous in eukaryotic cells; therefore, deficits in mitochondrial function, leading to reduced energy metabolism and oxidative capacity, can have drastic effects on highly metabolically active tissues such as the brain, skeletal muscle, the heart, the kidneys, and the liver (Fernandez-Moreno et al., 2000). According to Lopez-Lluch, Irusta, Navas, and de Cabo (2008), “effective control of mitochondrial biogenesis and turnover becomes critical for the maintenance of energy production, the prevention of endogenous oxidative stress and the promotion of healthy aging.” Elucidating the reasons behind mitochondrial functional decline and maintaining and improving mitochondrial function has become an increasingly studied area of interest.

Impaired mitochondrial biogenesis exerts “some of the most potent effects on the organism” and is implicated in the disease process of many prevalent diseases affecting the world’s population (Lopez-Lluch et al., 2008). In fact, defective mitochondria have to be
eliminated in order to maintain cellular homeostasis (Diaz & Moraes, 2008). Compromised mitochondrial biogenesis is notably involved in the development of the metabolic syndrome, also termed cardiometabolic syndrome, and subsequent risk and progression of both heart disease and type 2 diabetes, the number one and number eight leading causes of death, respectively, worldwide (WHO, 2013).

The metabolic syndrome is a composite of multiple metabolic disorders including obesity, hypertension, insulin resistance, and a prothrombotic and proinflammatory state (Nisoli et al., 2007; Ren et al., 2010). Metabolic syndrome has become more common among the United States population, affecting around 35% of adults, posing a problem with significant public health implications because of the “2-fold increased risk of prevalent coronary heart disease, 3- to 4-fold increased risk of mortality attributable to coronary heart disease, and a 6-fold risk of developing type 2 diabetes mellitus,” (AHA, 2011; Nisoli, Clementi, Carruba, & Moncada, 2007). Kim, Wei, and Sowers (2008) demonstrated that there are fewer and smaller-sized mitochondria in the skeletal muscle of insulin-resistant and obese subjects as well as decreased PGC-1α and NRF-1 expression in insulin-resistant and diabetic humans that was age-dependent, suggesting that mitochondrial dysfunction was largely attributable to decreased mitochondrial number and biogenesis. Indeed, when mitochondrial density was assessed in insulin-resistant subjects and compared with the mitochondrial density of control subjects, researchers found a 38% decrease in mitochondrial biogenesis among the insulin-resistant group (Morino et al., 2005). Obese subjects are “deficient in energy in the form of ATP,” causing an increase in appetite and a decreased exercise capacity (Nisoli et al., 2007). The resulting excess caloric intake promotes conditions, i.e. a high proton gradient and low oxygen consumption, that yield the highest production of ROS, leading to further mitochondrial and cellular damage (Kim et al.,
Insulin resistance has also been shown to decrease eNOS activity, decreasing mitochondrial biogenesis and causing mitochondrial dysfunction (Kim et al., 2008). Moreover, “defects in mitochondrial number and/or function are causally linked to the cardiovascular alterations found in obesity and the metabolic syndrome,” (Nisoli et al., 2007). Metabolic syndrome-associated mitochondrial dysfunction has also been implicated in the linked cardiovascular diseases, impairing cardiac functions that “may contribute to heart failure, cardiomyopathy, and coronary artery disease.” (Kim et al., 2008).

Mitochondria occupy 20% to 30% of cardiomyocyte cell volume; therefore, any deficits in their function can pose serious health risks to an individual (Kim et al., 2008). Nisoli, Clementi, Carruba, and Moncada (2007) assert “evidence is emerging to support the concept that alterations in mitochondrial biogenesis and energetics of cardiomyocytes are linked to the development and progression of cardiovascular disease and heart failure in obesity.” Mitochondrial number is increased during adaptive cardiac hypertrophy in order to meet the increased energy demands of the heart; however, when this compensatory mechanism is exceeded, “cardiac function deteriorates and shifts towards heart failure,” and the failing heart becomes “unable to maintain normal mitochondrial function due to down-regulation of the transcriptional pathway controlling mitochondrial biogenesis,” (Rimbaud et al., 2009). The finding that cardiac PGC-1α was decreased in an experimental model of heart failure supported the idea that mitochondrial dysfunction in cardiac disease was a result of down-regulated mitochondrial biogenesis. “Decreased mitochondrial biogenesis and oxidative capacity represent a hallmark of the failing heart. In this respect, energy metabolism and cardiac biogenesis are of therapeutic interest,” (Rimbaud et al., 2009). Mitochondrial biogenesis is a complex process critical to the maintenance of mitochondrial and, furthermore, cellular homeostasis; therefore,
insults to this system threaten cellular health and are implicated in many degenerative and age-related diseases.

Key lifestyle approaches to maintaining healthy mitochondria and promoting mitochondrial biogenesis include diet and exercise, both of which ultimately act to increase expression of the master regulator of mitochondrial biogenesis, PGC-1α. Numerous studies have shown that there is a positive correlation between endurance exercise and mitochondrial biogenesis, thereby increasing mitochondrial content in metabolically active tissues (Davies, Packer, & Brooks, 1981; Dudley, Abraham, & Terjung, 1982; Holloszy & Booth, 1976). However, the extent to which mitochondrial content is increased can be affected by the intensity and duration of exercise (Dudley, Abraham, & Terjung, 1982). The mitochondrial content of a tissue is dependent upon the energy demand of that tissue. For example, in their 1952 study, Paul and Sperling demonstrated that there were fewer mitochondria in sporadically working muscles of the back compared to the mitochondrial content of the heart, a continuously working muscle. It is also known that cold acclimatization in many different types of fish increases mitochondrial content of muscle cells (Siddel, 1998). Building upon this knowledge, Mitchell, Harris, Cordaro, and Starnes (2002) designed a study to explore core body temperature as an additional potential factor that could affect mitochondrial adaptations with chronic exercise (2002). After 9 weeks of training on a motor-driven treadmill, rats trained in an environment that prevented an elevation in core temperature had greater increases, almost two-fold, in mitochondrial biogenesis than rats trained in an identical manner, except at a higher core temperature. Increased cytochrome c oxidase levels, a key indicator of increased mitochondrial activity, confirmed these results (Mitchell, Harris, Cordaro, & Starnes, 2002). The animals trained in such a way do not get cold; however, they simply do not experience the exercise-induced rise in normal body temperature
that occurs normally, allowing researchers to explore the effect of core temperature on mitochondrial biogenesis with exercise.

As the mitochondria and their function become increasingly implicated in the disease process, the stimulation of mitochondrial biogenesis has become an attractive therapeutic target. It is well known that exercise training is beneficial in both preventing and treating the metabolic syndrome, obesity, type II diabetes, cardiovascular disease, and many other conditions, partly by increasing mitochondrial biogenesis. Mitchell et al.’s study (2002) provides compelling evidence that exercising in temperatures where core temperature is clamped at resting levels, exaggerating the increase in mitochondrial biogenesis seen with exercise, may be an even better treatment option for conditions with roots in metabolic dysfunction. Going a step further, the purpose of the study at hand was to explore the potential mechanisms of increased mitochondrial biogenesis in the cold-training model by examining the expression of proteins involved in the regulation of mitochondrial biogenesis. In particular, the amounts of PGC-1α, AMPK, eNOS, SIRT1, and SIRT3 were measured.

**Mitochondrial Biogenesis**

Mitochondrial biogenesis, defined as the growth and division of pre-existing mitochondria (Jornayvaz & Shulman, 2007), occurs when the tissue energy demand is raised to a level that puts stress on the ATP-producing capacity of pre-existing mitochondria (Chabi, Adhihetty, Ljubicic, & Hood, 2005). The process of mitochondrial biogenesis “increases net mitochondrial mass and functional capacity or helps preserve it by compensating for loss of damaged mitochondria,” (Weinberg, 2011). Environmental stresses including exercise, cold temperature, caloric restriction, and oxidative stress, which increase cellular energy demand, all act to trigger
mitochondrial biogenesis (Ventura-Clapier, Garnier, & Veksler, 2008). The genes involved in mitochondrial biogenesis are located in both nuclear and mitochondrial DNA, thereby necessitating carefully coordinated crosstalk between the two genomes for successful regulation of this key cellular process. Multiple pathways exist for the activation of mitochondrial biogenesis.

**Peroxisome Proliferator-activated Receptor γ Co-activator 1α (PGC-1α).** PGC-1α has been identified as the “master regulator” of mitochondrial biogenesis. It is a transcriptional coactivator that “increases expression of nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (mtTFA or TFAM), which in turn promote the expression, respectively, of nuclear and mitochondrial genes that are required for mitochondrial biogenesis,” (Brown, 2003). The amount of PGC-1α in cells is positively correlated with mitochondrial protein levels, mitochondrial mass, and oxidative capacity and is found to be higher in highly oxidative tissues, such as the heart and brown adipose tissue (Rimbaud, Garnier, & Ventura-Clapier, 2009). Physiological cues such as exercise, cold temperatures, and fasting activate β3-adrenergic receptors and increase intracellular AMP and Ca2+ concentrations to induce the expression of PGC-1α (Puigserver, P. et al., 1998; Boss, O. et al., 1999; Wu, H. et al., 2002). PGC-1α serves to link external stimuli with an internal metabolic response that helps the cell to meet increased energy demands (Austin & St-Pierre, 2012; Jornayvaz & Shulman, 2010). Previously, it has been shown that approximately 3-5% of electrons form superoxide, an oxygen free radical, during oxidative phosphorylation. It is possible, therefore, that an increase in mitochondria would result in a greater production of ROS; however, PGC-1α has also been shown to increase the expression of ROS-detoxifying enzymes, allowing cells to “benefit from increased respiration
without suffering from oxidative damage,” (Austin & St-Pierre, 2012). Other proteins including AMPK, eNOS, and SIRT1 have also been implicated in the regulation of mitochondrial biogenesis due to their convergence on PGC-1α and its corresponding activity (see schematic 1 below). While exercise has been shown to increase mitochondrial biogenesis through PGC-1α, it is unknown whether PGC-1α plays a role in the augmented mitochondrial biogenesis observed during exercise in which core temperature does not increase.

*Schematic 1: Regulators of mitochondrial biogenesis under examination*
**AMP-activated Protein Kinase (AMPK).** AMPK has been identified as a major regulator of mitochondrial biogenesis in response to chronic energy depletion in numerous studies (Hardie, 2004; Hardie & Sakamoto, 2006; Kahn *et al.* 2005). Because cellular AMP concentrations change more dramatically than either ADP or ATP, the level of AMP is a key indicator of the metabolic and energy status of the cell thus allowing AMPK to act as a molecular sensor of energy status (Hardie & Hawley, 2001; Jäger, Handschin, St-Pierre, & Spiegelman, 2007). As a result of AMPK stimulation, many of the cell’s catabolic processes are activated whereas many anabolic processes are suppressed (Hardie, Hawley, & Scott, 2006; Carling, 2004; Kahn, Alquier, Carling, & Hardie, 2005; Fryer & Carling, 2005). In response to a decrease in the ratio of ATP:AMP that occurs during exercise, AMPK is activated leading to the direct activation of PGC-1α by phosphorylation of serine and threonine residues (Jornayvaz & Shulman, 2010; Kim *et al.*, 2008).

The phosphorylation of PGC-1α by AMPK in low energy situations leads to PGC-1α activation and increased mitochondrial biogenesis (Jäger, S., Handschin, C., St-Pierre, J. & Spiegelman, B.M., 2007). In Bergeron et al. and Winder et al.’s studies (2001; 2000), pharmacologically-induced chronic activation of AMPK led to increased levels of mitochondrial biogenesis-related proteins and mitochondrial content. Multiple exercise studies also demonstrate that increased AMPK activity leads to increased PGC-1α mRNA and protein expression, resulting in upregulated mitochondrial biogenesis; although, exercise alone has been shown to be sufficient to increase PGC-1α mRNA expression independently of AMPK (Terada *et al.*, 2002; Atherton *et al.*, 2005; Jorgensen *et al.*, 2005). Therefore, measuring both AMPK and PGC-1α may provide some clues as to the mechanisms by which mitochondrial biogenesis is enhanced when exercising with core temperature clamped at resting values.
**Endothelial Nitric Oxide Synthase (eNOS).** Although largely responsible for endothelial-derived vasodilation, eNOS has also been found to play a role in the process of mitochondrial biogenesis. Shear stress serves to activate eNOS and increases during exercise, as working muscles require more oxygen, and thus more blood flow. Gielen et al. (2010) found that exercise increases eNOS phosphorylation at a specific serine residue, thereby increasing eNOS activity and nitric oxide (NO) production. The NO produced by eNOS acts on guanylate cyclase to increase cGMP levels. Through some unknown mechanism, this increased cGMP transduces a signal to the nucleus to induce the transcription of PGC-1α and subsequent mitochondrial biogenesis genes (Nisoli et al., 2003). It is interesting to note that high levels of NO inhibit many components of the respiratory chain, reversibly blocking mitochondrial respiration, whereas small amounts of NO stimulate mitochondrial biogenesis and improve the supply of oxygen and respiratory substrates to mitochondria (Moncada, 2002; Brown & Borutaite, 2002; Hibbs et al., 1987; Brown, 2003).

In their work, Nisoli et al. (2003) demonstrated that eNOS requires the induction of PGC-1α and the action of cGMP in order to induce mitochondrial biogenesis. When HeLa cells were manipulated to express eNOS, they exhibited an increase in PGC-1α, NRF-1, and TFAM mRNA expression, as well as increased mtDNA content and cytochrome c protein levels. The researchers also found that eNOS deletion was “sufficient to reduce mitochondria in brain, heart, and liver tissue of eNOS dominant-negative mice.” (Nisoli et al., 2003) Other studies have confirmed the detrimental effects of eNOS deficiency on metabolism, finding that eNOS-deficient animals displayed traits characteristic of the metabolic syndrome: reduced mitochondrial number, reduced energy expenditure, weight gain, insulin resistance, and hypertension (Shankar, Wu, Shen, Zhu, & Baron, 2000; Huang et al, 1995). To determine
whether the effect of core temperature on exercise-induced mitochondrial biogenesis was a result of increased eNOS expression, relative eNOS protein expression was determined in the different training paradigms of the study at hand (trained in room temperature vs. trained at 6-8°C).

**Silent Mating Type Information Regulator 2 Homolog 1 (SIRT1).** SIRT1 is one of seven mammalian NAD⁺-dependent deacetylases and ADP-ribosyltransferases belonging to the sirtuin family. Their dependence on NAD⁺ suggests that the family of sirtuins serve as sensors of the cellular metabolic and oxidative state (Imaai, Armstrong, Kaeberlein, & Giaremte, 2000). SIRT1 has typically been located in the nucleus and the cytoplasm (Haigas & Sinclair, 2010; Tanna, Sakamoto, Miura, Shimamoto, & Horio, 2007). However, according to Alquilano et al., copies of both SIRT1 and PGC-1α are also found near transcription factors in mitochondrial DNA (2010). Upon fasting/caloric restriction, pyruvate kinase induces the NAD-dependent deacetylase SIRT1, which was said to activate PGC-1α by deacetylation. (Jornayvaz & Shulman, 2010; Rodger\$ J.T. et al., 2005). Because the stress induced by caloric restriction is very similar to that caused by exercise, i.e. changes in metabolic and redox status, it has been hypothesized that exercise also increases SIRT1, although evidence for this has been inconclusive (Civitarese et al., 2007; Fulco et al., 2003; Lagouge et al., 2005; Suwa, Nakana, Radak, & Kumagai, 2008; Huffman et al., 2008; Philp et al., 2011).

The role of SIRT1 in mitochondrial biogenesis is up for debate, as numerous studies have yielded contradictory results concerning the relationship between SIRT1 and mitochondrial biogenesis. Numerous published studies report that SIRT1 deacetylation activates PGC-1α, whereas SIRT1 deacetylation deactivates PGC-1α (Suwa et al., 2008; Lagouge et al., 2006; Feige et al., 2008; Cantó et al., 2010; Bai et al., 2011; Gerhart-Hines et al., 2007; Lerin et al., 2006; Bai
et al., 2011; Coste et al., 2008; Kelly, Lerin, Haas, Gygi, & Puigserver, 2009). In 2013, Higashida et al. undertook a study to reevaluate the role of SIRT1 in mitochondrial biogenesis and its actions on PGC-1α. In summary, Higashida et al. (2013) found that PGC-1α is activated by acetylation, SIRT1 activity decreased PGC-1α activity, and the inhibition and knockdown of SIRT1 activity yielded increased PGC-1α activity. Other studies have also found evidence of an inverse relationship between SIRT1 protein content, SIRT1 mRNA, and mitochondrial content in both heart and skeletal muscle (Nemoto, Fergusson, & Finkel, 2005; Gurd, Yoshida, Lally, Holloway, & Bonen, 2009; Michisita, Park, Burneskis, Barret, & Horikawa, 2005; Shi, Wang, Stieren, & Tong, 2005). However, yet another study suggests that nuclear SIRT1 activity, not SIRT1 protein content, promotes mitochondrial biogenesis. Gurd et al. (2011), found that nuclear SIRT1 activity increased under the same conditions that stimulated mitochondrial biogenesis, such as pharmacological AMPK activation by AICAR and exercise, in both rat and human skeletal muscle. Interestingly, nuclear SIRT1 activity was inversely related to nuclear SIRT1 protein content. Taken together, Gurd et al.’s study (2011) suggests that mitochondrial biogenesis is inhibited when whole muscle SIRT1 protein is increased, but is stimulated when SIRT1 activity is high. However, none of the previously described studies evaluated the potential effect of core temperature on SIRT1-mediated mitochondrial biogenesis; therefore, we also measured SIRT1 expression in the present study.

**Silent Mating Type Information Regulator Homolog 3 (SIRT3).** Similar to SIRT1, SIRT3 is a NAD⁺-dependent deacetylase sensitive to the metabolic and redox state of the cell that has enhanced activity with exercise and caloric restriction (Onyango et al, 2002; Sack, 2011; Palacios et al., 2009). Unlike SIRT1, however, SIRT3 is localized to the cristae of the mitochondria. Due to its location in the mitochondria, SIRT3 expression is greatest in
metabolically active tissues such as the kidney, brown adipose tissue, and the heart (Onyango et al., 2002). SIRT3 acts to promote acetate and fatty acid catabolism through the deacetylation of various mitochondrial enzymes, including acetyl Co-A (Hallows et al., 2006; Schwer et al., 2006; Guarente, 2011). In addition to its role in mitochondrial metabolism and respiration, SIRT3 also plays a role in the response to oxidative stress. Notably, SIRT3 deacetylates the key antioxidant enzyme manganese superoxide dismutase (SOD2), increasing its antioxidant activity and ROS scavenging (Qiu et al., 2010; Tao et al., 2010). As discussed earlier, PGC-1α has been linked to the response to oxidative stress. In their 2010 study, Kong et al. found that SIRT3 is a target of PGC-1α. The activation of SIRT3 by PGC-1α is mediated by ERRα, an orphan nuclear hormone receptor. In addition to its ROS-detoxifying abilities, SIRT3 also stimulated PGC-1α expression through the phosphorylation of CREB, thereby creating a positive-feedback loop. Even more, the researchers found that inhibition of SIRT3 expression reduced PGC-1α-induced mitochondrial biogenesis, although the molecular mechanism is unknown (Kong et al., 2010). As a potential regulator of mitochondrial biogenesis, SIRT3 was measured in the study at hand to elucidate the pathways by which core temperature may affect the mitochondrial biogenesis that accompanies endurance exercise.

Methods

Animals

Female, 5-month-old, Sprague-Dawley rats were purchased and housed in an isolated room in the University of North Carolina Greensboro campus animal facility. Animals were kept on a 12:12 hour light-dark cycle and fed ad libitum with Harlan 2018 Teklad Global 18% Protein Rodent Diet. Rats were randomly divided into one of the following three treatment groups:
sedentary control (SED; n=10), exercised in a 22°C room (ET; n=10), and exercised in the cold (E; n=11). Core temperatures were held constant in the cold exercise group by maintaining room temperature between 6-8°C. These conditions were designed to maintain exercising core temperatures (T<sub>C</sub>) at 40.4°C or at a normal resting temperature of 38.0°C, respectively. Experiments were approved by the University of North Carolina Greensboro’s Animal Care and Use Committee and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Training**

Animal training was carried out by our collaborators at the University of North Carolina, Greensboro. Exercise was carried out on a Collins 12-lane motor-driven rodent treadmill equipped with a Coulbourne Precision Regulated Animal Shocker at the back to encourage animals to run in the correct direction. Very low current (0.5 ams) was used and voltage kept at a minimum (10-30 volts) to keep the animals running as described in the Resource Book for the Design of Animal Exercise Protocols, published by The American Physiological Society in 2006. All exercising groups were initially habituated to the treadmill at room temperature by gradually increasing running time and speed each day so that at the end of one week they were running for 15 minutes at 20 meters/min and 25 meters/min for the second 15 minutes up a 6% grade. Following habituation, the cold training group began to run in temperatures between 6-8°C to prevent an excessive increase in core temperature. The duration of each exercise bout was gradually increased to 60 min/day for 5 days/week by the end of 3 weeks. Speed of exercise was gradually increased so that at the end of the 5-week training regimen rats were running for 1 hour per day at 30 meters/min for 5 days/week (See Appendix A for rat training schedule). It was
previously determined that this exercise intensity is ~70% of the maximum oxygen consumption ($VO_{2\text{max}}$) in the untrained state (Bowles et al, 1991). All animals were housed at 22°C when not exercising. Animals that ran in the cold were returned to a 22°C room immediately after exercise. Throughout the exercise programs, $T_C$ was monitored periodically with a digital thermocouple thermometer to assure consistency. During brief rest periods at 15-min intervals, the probe was inserted 5 cm into the rectum in order to measure the $T_C$. Trained animals rested for 24 hours after the last exercise bout to remove acute training effects and then killed in order to harvest tissues, as described below.

**Tissue Preparation**

Animals were euthanized by opening the chest cavity under anesthesia with rodent anesthesia at a dosage of 0.7 ml/kg administered intraperitoneally. The composition of the cocktail was 100 mg/ml ketamine, 20 mg/ml xylazine, and 10 mg/ml Acepromazine. Whole plantaris muscles were excised, wrapped in aluminum foil and stored -100°C for later analysis. The tissues were then thawed, weighed, and homogenized in 20 volumes (1:20 wt/vol) of 50 mM KH$_2$PO$_4$, 0.1 mM EDTA, 0.1% (vol/vol) Triton X-100, pH 7.4 with a Protease Inhibitor Cocktail (Sigma No. P8340) in a Teflon-glass Potter-Elvehjem homogenizer. Samples were centrifuged at 10,000xg for 5 minutes and the supernatants analyzed for protein concentration by the Lowry method.

**Cytochrome-c Oxidase Assay**

Cytochrome-c Oxidase (CytOx), the final protein complex in the mitochondrial electron transport chain, was used as an indicator of the number of electron transport chains present in the
muscle. Plantaris muscle was homogenized and treated with Triton X-100 (0.1% vol/vol). Triton X-100-treated homogenate (30 µl) was added to 1.47 ml of assay medium containing (in mM) 50 K2 HPO4, 0.1 EDTA, 0.62 tetramethylpentadecane, 12.5 sodium ascorbate, and 0.04 cytochrome c, pH 7.4. CytOx activity was then measured polarographically with a Clark-type oxygen electrode at 25°C, as described by Rumsey et al. (1987).

Immunoblotting

Approximately, 60 µg of each sample were loaded and run on a 10% polyacrylamide gel and transferred to a Whatman Protran nitrocellulose membrane, which underwent subsequent immunoblotting according to the manufacturer’s instruction for Western Blot Analysis for the Odyssey imager (LI-COR; 2012). The following primary antibodies were used to detect protein expression: SIRT1 anti-mouse (sc-74465, Santa Cruz Biotechnology; dilution 1:500), Hsp70 anti-mouse (sc-66048, Santa Cruz Biotechnology; dilution 1:500), β-actin anti-mouse (sc-47778, Santa Cruz Biotechnology; dilution 1:500), SIRT3 anti-mouse (sc-365175, Santa Cruz Biotechnology; dilution 1:500), eNOS anti-mouse (610297, BD Biosciences; dilution 1:500), AMPK anti-rabbit (2532, Cell Signaling Technology; dilution 1:500), GLUT4 anti-mouse (2213, Glut4; dilution 1:1000), PGC-1α anti-rabbit (sc-13067, Santa Cruz Biotechnology; dilution 1:200). Results from immunoblotting were determined by densitometric analysis of the fluorescent bands using the LI-COR Odyssey Sa imager (Model #9260). Densities for each sample will be normalized by calculating a ratio of density of the sample to a standard sample homogenate created from the plantaris of a sedentary rat, which was loaded on each blot.

Statistical Analysis
The reported values represent means ± standard error (SE). To determine whether there was a significant difference among groups after 5 weeks of training at different temperatures, one-way ANOVA with Newman-Keuls post-hoc tests were used to analyze the results from the densitometric analysis of the immunoblots. A P-value of <0.05 was used as a limit for statistical significance.

Results

CytOx activity in the plantaris muscles of animals exercised for 5 weeks at various core temperatures is displayed in Fig 1. Both exercising groups had higher CytOx activity than the sedentary group (mean= 24.16 ± 1.37 µmoles O$_2$·min$^{-1}$·g wet wt$^{-1}$), and the magnitude of the increases was related to the exercise T$_{C}$. The CytOx activity of animals exercising in a typical room temperature of 22°C with an exercising T$_{C}$ of 40.4°C was significantly greater (ET= 37.00 ±2.09; P<0.05) than the CytOx activity of the SED group. When exercise conditions were further altered to keep T$_{C}$ clamped at resting temperature (T$_{C}$= 38°C), CytOx activity was also significantly elevated in the E group (E= 47.14 ± 4.11, P< 0.05) compared to the SED group. Newman-Keuls post-hoc test revealed that the CytOx activity of the E rats (T$_{C}$= 38°C) was also significantly (P<0.05) greater than that of the ET rats (T$_{C}$=40.4°C). Having confirmed the exaggerated increase in mitochondrial biogenesis seen with exercise absent of any increase in core temperature, we continued to measure the expression of various proteins involved in the regulation of mitochondrial biogenesis to elucidate potential mechanisms for this response.

As a measure of the effect of temperature, analysis of Hsp70 expression was measured. Densitometric analysis of the rat plantaris muscle immunoblots revealed a significant (P<0.05)
increase in Hsp70 expression in both the room temperature trained rats (ET=2.98 ± 0.24) and the cold-trained rats (E= 1.64 ± 0.19) versus sedentary rats (SED= 0.81 ± 0.05; Fig 2). As expected, Hsp70 expression was also significantly (P< 0.05) higher in the ET rats versus the E rats (Fig 2). Additionally, serum lactate dehydrogenase (LDH) was measured as an indicator of stress and tissue damage. Our data shows a significant (P<0.05) increase in serum LDH in the plantaris muscles of room temperature trained rats compared to both the sedentary and cold-trained rats (Fig 3).

Analysis of immunoblotting results using one-way ANOVA with Newman-Keuls post-hoc tests revealed that there were no significant differences in PGC-1α expression in either of the trained groups (ET=0.82 ± 0.05; E=0.88 ± 0.06) versus the sedentary group (SED=0.92 ± 0.06; Fig 4). AMPK expression was not significantly altered in neither the room temperature-trained rats (ET=1.03 ± 0.13) nor the cold-trained rats (E=1.07 ± 0.13) compared to the sedentary rats (SED=1.15 ± 0.08; Fig 5). One-way ANOVA with Newman-Keuls post-tests also revealed that there were no significant differences in eNOS protein expression among the sedentary and trained groups, although eNOS expression in ET (ET= 1.96 ± 0.41) and E (E=1.88 ± 0.35) groups tended to be higher than the SED group (SED=1.59 ± 0.29; Fig 6). SIRT1 expression was significantly (P< 0.05) decreased in the plantaris muscles of the rats trained at room temperature for 5 weeks (ET=0.79 ± 0.05) versus both the sedentary and 5-week cold-trained rats (SED=1.02 ± 0.05 and E=0.94 ± 0.05; Fig 7). Although SIRT1 expression appears to be lower in the E group compared to the SED group, the difference is not significant (Fig 7). One-way ANOVA analysis with Newman-Keuls post-hoc tests revealed that SIRT3 expression was significantly (P< 0.05) elevated among both the ET (ET= 2.07 ± 0.12) and E (E= 2.15 ± 0.15) groups compared to the SED (SED= 1.64 ± 0.12) group (Fig 8).
Discussion

Mitochondrial dysfunction has been identified in the etiology of many prevalent diseases, including the metabolic syndrome, diabetes, and associated cardiovascular disease (Kim et al., 2008; Coste et al., 2008; Nisoli et al., 2007; Ren et al., 2010; Morino et al., 2005; Rimbaud et al., 2009). Endurance exercise is important for prevention, risk reduction, and treatment of these diseases, partly through the enhancement of mitochondrial function and biogenesis (Davies et al., 1981; Dudley, et al., 1982; Holloszy & Booth, 1976). A previous study done by Mitchell et al. (2002) found that mitochondrial biogenesis increased, nearly double, in exercising conditions where core temperature remained constant. This cold-training model isolates the effects of mechanical stimulation from the effect of mechanical stimulation coupled with an increase in core temperature typically seen with exercise. The current study sought to elucidate potential mechanisms whereby this augmented mitochondrial biogenesis occurred, evaluating the effect core temperature may have on the process of mitochondrial biogenesis at the molecular level. Our data indicates that mitochondrial biogenesis in the cold-training model occurs by a different pathway than it does in normal exercise.

As expected, the results of the CytOx activity assay indicates that increased mitochondrial biogenesis occurred in the tissue of trained rats. Aligning with previous results, CytOx activity in the present study was higher in the cold-trained rats compared with the room temperature trained rats, validating the training model of the Mitchell et al. (2002) study (Fig 1). However, Hsp70 was significantly elevated not only in the tissues of room temperature trained rats but also in the tissues of cold-trained rats (Fig 2). This indicates a slight variation from the findings of the previous study, where Hsp70 was elevated only in the room temperature trained
rats. This variance may be due to differences in the training regimens used, for instance the animals ran harder for a shorter period of time in the present study compared to the previous study, which may have caused their core temperatures to increase more than expected. Another potential reason for the disparity in results is the difference in gender between the two studies. In addition, it should be noted that the study by Mitchell et al. (2002) also reported an elevation in mitochondrial Hsp70 (mtHsp70) that correlated with CytOx activity in cold-trained rats. Therefore, it appears that the mtHsp70 is related more to changes in mitochondrial content than to increases in temperature. This may also explain the increase in total Hsp70 in the present study using an antibody that did not distinguish between the two isoforms, Hsp70 and mtHsp70. Thus, the increase in whole-muscle Hsp70 expression in cold-trained rats in the present study may be partially accounted for by an increase in mtHsp70 as well. In their study, Samelman, Shiry, and Cameron (2000) rationalized that mtHsp70 has two functions: the majority serves as a matrix unfoldase, but a small fraction is involved with the transport of nuclear-encoded subunits into the mitochondria. This additional role of mtHsp70 suggests a positive relationship between mitochondrial biogenesis and Hsp70 content, which would align with our results. In future studies, both mtHsp70 and Hsp70 should be measured separately.

The present study went a step further to evaluate muscular stress/damage by analyzing serum lactate dehydrogenase (LDH). LDH is a marker for tissue damage and injury and is found in many body tissues, including but not limited to the muscles, heart, brain, and kidney (NIH, 2012). Because serum LDH was significantly higher in the tissues of room temperature trained rats, our study indicates that cold training is far less stressful to the organism than normal exercise (Fig 3). Perhaps this is due to heat stress-induced ROS production, which has been reported to increase the production of ROS possibly by interfering with electron transport.
assemblies of the inner mitochondrial membrane (Ando et al., 1997; Bruskov et al., 2002; Mujahid et al., 2005). An increased threat to protein structure and increased protein unfolding known to occur with increases in temperature may also account for the difference. Although it is not clear exactly why cold training is less stressful, preventing the increase in core temperature seen with exercise appears to be protective. Importantly, as part of a treatment regimen for diabetes, CVD, or the metabolic syndrome, the cold-training model may be preferred to normal exercise because it is less stressful to the organism.

Although there are many studies that report a positive correlation between PGC-1α expression and exercise, the results of the present study indicate that there was no significant increase in PGC-1α expression in the plantaris tissues of either room temperature- or cold-trained samples versus the sedentary sample (Rimbaud, Garnier, & Ventura-Clapier, 2009; Puigserver, P. et al., 1998; Boss, O. et al., 1999; Wu, H. et al., 2002; Austin & St-Pierre, 2012; Jornayvaz & Shulman, 2010) (Fig 4). This discrepancy may be due to the type of muscle evaluated. A study by Suwa et al. (2008) found that PGC-1α expression was differential across muscle fiber types. They observed an increase in mitochondrial biogenesis with endurance exercise in both plantaris, a type-2, fast-twitch muscle, and soleus, a type-1, slow-twitch muscle; however, an increase in PGC-1α expression was absent among the fast-twitch plantaris tissues but present in the slow-twitch soleus tissues, which is consistent with our observations (Suwa et al., 2008). A limitation of the present study, therefore, is that we only measured PGC-1α expression in plantaris tissues. It would be beneficial to include other muscle types in future studies to better understand the role PGC-1α plays in the exaggerated mitochondrial biogenesis seen in the cold training model, but the present study suggests its role may be limited in exercise-induced mitochondrial biogenesis in fast-twitch fibers like the plantaris. It is also possible that while PGC-1α expression was not
elevated, PGC-1α activity may have been upregulated in the training models. Since it has been found that phosphorylation by AMPK activates PGC-1α, future studies should include a measurement of PGC-1α activity (Jäger et al., 2007).

Despite evidence that AMPK is a major regulator of mitochondrial biogenesis through the direct activation of PGC-1α, the present study also found no significant change in AMPK expression between the training models and the sedentary controls (Fig 5) (Hardie, 2004; Hardie & Sakamoto, 2006; Kahn et al. 2005). The results of the current study suggest that there is another protein responsible for the exaggerated increase in mitochondrial biogenesis when core temperature is clamped during endurance exercise. Once again the question of protein expression versus protein activity comes into play, and it should be addressed in future studies by including a measurement of AMPK activity. Similarly, no significant change in eNOS expression among any of the training groups studied was found in the present study, suggesting that eNOS does not likely play an important role in exaggerated mitochondrial biogenesis with cold-training (Fig 6). Previously, Nisoli et al. (2003) found that eNOS plays a role in the regulation of mitochondrial biogenesis through the production of NO and subsequent activation of its secondary messenger cGMP. Perhaps eNOS activity, rather than eNOS content, may be related to mitochondrial biogenesis. Therefore, it may be useful to measure phospho-eNOS to examine the effect of eNOS activity. Also, NO could come from other sources, such as nNOS or iNOS, a limitation of the present study and one that should be considered in future studies.

The results of the present study align with the Higashida et al. (2013) study concerning the role of SIRT1 in exercise-induced mitochondrial biogenesis. SIRT1 expression was significantly decreased in tissues subjected to a room temperature training regimen, suggesting that SIRT1 may be a negative regulator of exercise-induced mitochondrial biogenesis (Fig 7). A
relationship between SIRT1 and tissue stress may be a possible explanation, although the
directionality is not clear. The decrease in SIRT1 expression observed in tissues of room
temperature trained rats may make organisms more susceptible to stress, and, therefore, serum
LDH was elevated in these same rats. On the other hand, increased serum LDH and tissue
damage may inhibit SIRT1 expression. Because SIRT1 expression rose back to baseline levels in
the plantaris muscles of cold-trained rats compared to room temperature trained rats, it appears to
not play a role in the amplified response to cold training (Fig 7). One reason for this may be that
the cold-trained rats adapt more quickly to the stress of exercise than do the normal exercisers; it
may be that SIRT1 initially decreases with exercise but returns to baseline levels as animals
adapt. In the future, measurements of SIRT1 expression at different time points within the
exercise regimen would potentially provide evidence for such a time-dependent relationship
between SIRT1 expression and exercise. Another possibility is that SIRT1 expression varies with
tissue type. Our present study only looked at plantaris muscle samples, but a study done by Suwa
et al. (2008) found that SIRT1 expression appears to be higher in the soleus muscle, a type-1,
slow-twitch muscle, than in the plantaris muscle, a type-2, fast-twitch muscle. This represents a
limitation in the present study, and in the future it would provide more insight into the role of
SIRT1 in mitochondrial biogenesis to measure its expression across various tissue types,
including red, slow-twitch, oxidative muscle and cardiac muscle.

A previous study by Kong et al. (2010) found that SIRT3 may be a target of PGC-1α.
Furthermore, the researchers found that SIRT3 provided positive feedback on PGC-1α
expression, suggesting SIRT3 as a regulator of mitochondrial biogenesis. Similarly, in our study,
SIRT3 was significantly increased in both room temperature and cold-trained rats, suggesting a
positive relationship between SIRT3 expression and mitochondrial biogenesis (Fig 8) While
there is compelling evidence for SIRT3 as a regulator of mitochondrial biogenesis (Kong et al., 2010), our data do not indicate an augmented role for SIRT3 in the exaggerated mitochondrial biogenesis resulting from clamping core temperature during exercise. Because SIRT3 expression was similar among training groups, regardless of core temperature, some other mechanism must be involved in the augmentation of mitochondrial biogenesis (Fig 8). Another possibility for the similar expression of SIRT3 across training groups is that SIRT3 may be similar to SIRT1 in that its expression could be time-dependent. Since cold training was less stressful, the cold trainers may have adapted more quickly than the other training group. It is possible that SIRT3 expression rose dramatically in the cold-training group during the 5-week exercise-training regimen and then fell to a level comparable to that of the room temperature trained rats once they adapted. Again, it would be helpful to include measurements of SIRT3 expression throughout the training program to elucidate whether such a relationship exists.

Taken together, the results of our data suggest that both room temperature- and cold training-induced mitochondrial biogenesis takes place by a mechanism different from the currently accepted model of mitochondrial biogenesis with exercise (see Fig 9). Our data align with the findings of Higashida et al. (2013) regarding the much-debated role of SIRT1 in mitochondrial biogenesis: SIRT1 is not involved or is negatively correlated with the process of mitochondrial biogenesis. While we affirmed Mitchell et al.’s (2002) finding that preventing a rise in core temperature elicits exaggerated mitochondrial biogenesis in response to exercise, the exact mechanism by which this augmented response occurs is still unclear. Unique from other studies, we found no correlation between the expression of PGC-1α, AMPK, or eNOS and mitochondrial biogenesis. While SIRT3 expression was significantly elevated in the cold-trained rats, it was not any different from the expression of SIRT3 in response to exercise at room
temperature. It is important to note that the data from the present study only reflect a snapshot of the process of mitochondrial biogenesis and that it may be a dynamic process that changes over time. To provide evidence for these proteins as the potential regulators responsible for the increase of mitochondrial biogenesis in response to the cold training model, it would be ideal to perform a future study in which cold training-induced mitochondrial biogenesis was studied in animals with inhibited PGC-1α, AMPK, eNOS, SIRT1, and SIRT3 by pharmacologic or genetic methods. The finding that cold training was significantly less stressful than normal exercise, evidenced by a significantly (P<0.05) lower serum LDH level, suggests that this model of training, one in which a rise in core temperature is prevented, may be a better treatment option for those suffering from diseases known to be improved by exercise, namely diabetes, metabolic syndrome, or cardiovascular disease.

The outlook for research aimed at targeting mitochondrial function as therapeutic agents to prevent the decline of cellular function and age-related diseases is promising. The strategy of inducing mitochondrial biogenesis to produce healthy, fully functional mitochondria and the elucidation of various components that play a role in this process have created a set of therapeutic targets for the treatment and prevention of many metabolic and aging-related diseases. While the mechanism responsible for the augmented mitochondrial biogenesis observed with exercise at temperatures where core temperature remains at baseline levels remains unclear, the potential benefits of cold training should be considered when looking into treatments for pathologies with foundations in metabolic dysfunction; however, it may not yet be time to take the “polar plunge.”
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Interaction of temperature and exercise on cytochrome-c oxidase. CytOx activity is elevated with exercise training in rat plantaris muscle of sedentary (SED; n=11), 5-week room temperature trained rats (ET; n=11), and 5-week cold-trained rats (E; n=11). *Significant difference between SED and ET and SED and E (P<0.05). † Significant difference between ET and E (P<0.05). Values are means ± SE.
**Figure 2**

A. 

**Hsp70 expression in rat plantaris muscle**

![Graph showing Hsp70 expression](image)

**B.**

**Hsp70 expression is elevated with exercise training.** (A) Hsp70 expression relative to total protein concentration and standardized to sedentary sample in rat plantaris tissue of sedentary (SED; n=10), 5-week room temperature trained rats (ET; n=10), and 5-week cold-trained rats (E; n=11). *Significant difference between SED and ET and SED and E (P<0.05). † Significant difference between ET and E (P<0.05). (B) Western blot image of Hsp70 expression. Values are means ± SE.
**Figure 3**

**Serum LDH levels**

Exercise at room temperature leads to elevated serum LDH levels. Serum LDH is elevated with exercise training at room temperature in rat plantaris muscle of sedentary (SED; n= 13), 5-week room temperature trained rats (ET; n=9), and 5-week cold-trained rats (E; n=8).

*Significant difference between SED and ET and ET and E (P<0.05).
Figure 4

A. PGC-1α expression is unchanged with exercise training. (A) PGC-1α expression relative to total protein concentration and standardized to sedentary sample in rat plantaris tissue of sedentary (SED; n=10), 5-week room temperature trained rats (ET; n=10), and 5-week cold-trained rats (E; n=11). (B) Western blot image of SIRT1 expression. Values are means ± SE.
Figure 5

A. AMPK expression is unchanged with exercise training. (A) AMPK expression relative to total protein concentration and standardized to sedentary sample in rat plantaris tissue of sedentary (SED; n=10), 5-week room temperature trained rats (ET; n=10), and 5-week cold-trained rats (E; n=11). (B) Western blot image of SIRT1 expression. Values are means ± SE.
Figure 6

A. eNOS expression with exercise training. (A) eNOS expression relative to total protein concentration and standardized to sedentary sample in rat plantaris tissue of sedentary (SED; n=10), 5-week room temperature trained rats (ET; n=10), and 5-week cold-trained rats (E; n=11).

B. Western blot image of SIRT3 expression. Values are means ± SE.
SIRT1 expression with exercise training. (A) SIRT1 expression relative to total protein concentration and standardized to sedentary sample in rat plantaris tissue of sedentary (SED; n=10), 5-week room temperature trained rats (ET; n=10), and 5-week cold-trained rats (E; n=11). *Significant difference between SED and ET and E and ET (P<0.05). (B) Western blot image of SIRT1 expression. Values are means ± SE.
**Figure 8**

A.

**SIRT3 expression in rat plantaris muscle**

![Bar chart showing SIRT3 expression in different training groups](image)

**B.**

![Western blot image of SIRT3 expression](image)

**SIRT3 expression is elevated with exercise training.** (A) SIRT3 expression relative to total protein concentration and standardized to sedentary sample in rat plantaris tissue of sedentary (SED; n=10), 5-week room temperature trained rats (ET; n=10), and 5-week cold-trained rats (E; n=11). *Significant difference between SED and ET and SED and E (P<0.05). (B) Western blot image of SIRT3 expression. Values are means ± SE.
Differences between room temperature and cold training models. The figure above depicts a summary of the results found in the present study regarding the pathway by which exaggerated mitochondrial biogenesis occurs in the cold training model.
Appendix A

Below is the daily procedure for the rat protocol we used. We ran some at 22°C and some at 6-8°C.

**Week 1 – at room temperature (Habituation)**
- Day 1 – 15 min at 15 m/min at 6° grade
- Day 2 – 25 min at 20 m/min
- Day 3 – 25 min at 20 m/min; 5 min at 25 m/min
- Day 4 – 20 min at 20 m/min; 10 min at 25 m/min
- Day 5 – 15 min at 20 m/min; 15 min at 25 m/min

**Week 2 – at 8°C, refrigerated room**
- Day 6 – 20 min at 20 m/min; 10 min at 25 m/min
- Day 7 – 30 min at 25 m/min
- Day 8 – 35 min at 25 m/min
- Day 9 – 40 min at 25 m/min
- Day 10 – 45 min at 25 m/min

**Week 3 – at 8°C, refrigerated room**
- Day 11 – 50 min at 25 m/min
- Day 12 – 55 min at 25 m/min
- Day 13 – 60 min at 25 m/min
- Day 14 – 60 min at 26 m/min
- Day 15 – 60 min at 27 m/min

**Week 4 – at 8°C, refrigerated room**
- Day 16 – 60 min at 28 m/min
- Day 17 – 60 min at 29 m/min
- Day 18 – 60 min at 30 m/min
- Day 19 – 60 min at 30 m/min
- Day 20 – 60 min at 30 m/min

**Week 5 – at 8°C, refrigerated room**
- Day 21 – 60 min at 30 m/min
- Day 22 – 60 min at 30 m/min
- Day 23 – 60 min at 30 m/min
- Day 24 – 60 min at 30 m/min
- Day 25 – 60 min at 30 m/min