2015 Ph.D Dissertation

Curcumin Treatment Regulated Mitochondrial Biogenesis by Increasing Cyclic Adenosine Monophosphate (cAMP) Level in Rat Skeletal Muscle

Graduate School of Natural Science and Technology
Division of Life Sciences
Kanazawa University

1223032010
Hamidie Ronald Daniel Ray

Table of Contents

Abbreviations

ADP : adenosine diphosphate

AICAR : 5-aminoimidazole-4-carboxamide ribonucleoside

AMPK : adenosine monophosphate activated protein kinase

ATP : adenosine triphosphate

ATP6 : adenosine triphosphate synthase F0 subunit 6

Acs : adenylyl cyclases

5′ AMP : 5′ adenosine monophosphate

AKAPs : A kinase anchoring proteins

ATF1 : cyclic adenosine monophosphate-dependent transcription factor ATF-1

cAMP : cyclic adenosine monophosphate

cGMP : cyclic guanosine monophosphate

β-GPA : beta-guanadinopropionic acid

CaMKIV : calcium/calmodulin-dependent protein kinase IV

COX : cytochrome c oxidase

COX-IV : cytochrome c oxidase complex IV subunit IV

CREB : cyclic adenosine monophosphate response element binding protein

DMSO : dimethyl Sulfoxide

DNA : deoxyribonucleic acid

DRP1 : dynamin-related proteins 1

DTT : dithiothreitol

DTNB : 5,5-dithiobis (2-nitrobenzoate)

ECL : enzymatic chemiluminescence

EDTA : ethylene diamine tetraacetic acid

EGTA : ethylene glycol tetraacetic acid

ETC : electron transport chain

ERRα : estrogen related receptor alpha

eTR : endurance training

FoxO : forkhead homeobox type O

GAPDH : glyceraldehydes-3-phosphate dehydrogenase

Gas : m. gastrocnemius

GLUT4 : glucose transporter 4

Hsp70 : heat shock proteins 70

HRP : horseradish peroxidase

IMF : intermyofibrillar

LKB1 : liver kinase B1

mtDNA : mitochondrial deoxyribo nucleic acid

mRNA : messenger ribonucleic acid

MMP : mitochondrial membrane potential

Mfn1 / 2 : mitofusins 1 / 2

MEF2A : myocyte-specific enhancer factor 2A

MO25 : mouse protein-25

MMPs : matrix metalloproteinases

NaF : sodium (natrium) floride

Nampt : nicotinamide phosphoribosyltransferase

NAD^+ : nicotinamide adenine dinucleotide

ND1 : NADH dehydrogenase 1

nDNA : nuclear DNA

NRF-1/2 : nuclear respiratory factor ½

OAA : oxaloacetate

OPA1 : optic atrophy 1

OXPHOS : oxidative phosphorylation

PARP-1 : poly Adenosine diphosphate-ribose polymerase-1

PDE : phosphodiesterase

PKA : protein kinase A

PKC : protein kinase C

PMSF : phenylmethylsulfonyl fluoride

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

ROS : reactive oxygen species

PRC : peroxisome proliferator-activated receptor gamma coactivator related coactivator

RNA : ribonucleic acid

RT-PCR : real time polymerase chain reaction

SDS-PAGE : SDS-polyacrylamide gel electrophoresis

STRAD : sterile-20-related adaptor

SIRT1 : sirtuin 1

SAMP8 : senescence-accelerated mouse-prone 8

Sol : m. soleus

SS : subsarcolemmal

Tfam : mitochondrial transcription factor A

TIM : translocase of the inner membrane

TNF-alpha : tumor necrosis factor alpha

TOM : translocase of the outer membrane

ZMP : 5-amino-4-imidazolecarboxamide ribotide

List of Figures

List of Table

This dissertation consists of the results of the following publications and unpublished experimental data.

(1) Hamidie, R. D. R., Yamada, T., Ishizawa, R., Saito, Y. and Masuda, K. (2015) Curcumin treatment enhances the effect of exercise on mitochondrial biogenesis in skeletal muscle by increasing cAMP levels. Metabolism. 64: 1334-1347

I. Introduction

The skeletal muscle is one of the largest organs in the body, and it has great adaptive potential in response to physiological stressors. A marked example of muscle adaptation occurs in the mitochondria following exercise training. The biogenesis of mitochondria and the clearance of damaged mitochondria can promote healthy muscle. This turnover can prevent imbalances in metabolism, which can predispose individuals to the development of obesity, diabetes, cardiovascular disease, and accelerated aging (Joseph et al, 2012; Patti et al, 2003). Endurance exercise training has the potential to enhance metabolic characteristics in the skeletal muscle, including mitochondrial biogenesis and glucose transporter 4 (GLUT4) expression (Holloszy, 2008). The mechanisms underlying such adaptations remain to be elucidated, although such adaptations have been investigated for several decades. Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) has been implicated as a master regulator of mitochondrial biogenesis via its interaction with nuclear respiratory factor 2 (NRF-2/GA-binding protein-A) and nuclear respiratory factor 1 (NRF1). Both PGC-1α and NRF-1 activate the mitochondrial transcription factor A (Tfam), which is responsible for transcribing nuclear-encoded mitochondrial genes, as well as proteins involved in mitochondrial DNA (mtDNA) transcription, translation, and repair. The PGC-1α mRNA and protein expression levels are increased by acute endurance exercise and endurance exercise training

(Russell et al., 2003; Taylor et al., 2005), thus suggesting that PGC-1α is a potential regulator of metabolic adaptations with endurance exercise.

Mammalian sirtuins, such as sirtuin 1 (SIRT1), are members of a conserved family of NAD^+ (Nicotinamide adenine dinucleotide)-dependent deacetylases and ADP (adenosine diphosphate)-ribosyltransferase, which are involved in numerous fundamental cellular processes, including gene silencing, DNA (deoxyribo nucleic acid) repair, and metabolic regulation. SIRT1 promotes mitochondrial biogenesis via the deacetylation of PGC-1α. Another important metabolic sensor, AMP-activated protein kinase (AMPK), also activates PGC-1α. The expression and activity of SIRT1 in rat skeletal muscle (Ferrara et al., 2008) have been observed to increase with endurance exercise. Similar to AMPK, SIRT1 is widely associated with energy metabolism, thus implicating mitochondrial function, and activators of these enzymes could enhance mitochondrial biogenesis and endurance capacity. Collectively, these findings suggest the possibility that the metabolic adaptations resulting from endurance exercise training result, at least partly, through increased PGC-1α protein expression through AMPK pathways. The NAD^+-dependent histone deacetylase SIRT1 is also activated by acute endurance exercise (Suwa et al., 2008). Thus, it has been proposed that AMPK activates SIRT1 indirectly by increasing the intracellular levels of its co-substrate, NAD^+.

In both mammalian cells and yeast, the regulation of mitochondrial biogenesis clearly involves the cAMP (cyclic adenosine monophosphate) signaling pathway. Indeed, it has been shown that treatment of human

preadipocytes with forskolin, which leads to an overactivation of the cAMP pathway, increased mitochondrial DNA copy number (Bogacka et al., 2005). Protein kinase A (PKA), a well studied cAMP downstream effector is also known as cAMP-dependent enzyme because it gets activated only if cAMP is present. Once PKA is activated, it phosphorylates a number of other proteins including cAMP response element binding protein (CREB) and liver kinase B1 (LKB-1) which induction PGC-1α to regulated mitochondria biogenesis (Than et al., 2011; Veeranki et al., 2011). Previous study shown evidence that exercise included swimming and running increased cAMP on skeletal muscle and myocardium (Palmer, 1988). This evidence together strong suggested that exercise increased mitochondrial biogenesis through its ability to increase cAMP. In This present study we examined effect of curcumin treatment on cAMP level and downstream target of PKA included phosphorylation CREB and LKB-1 which involved on mitochondrial biogenesis.

Several polyphenols have been shown to activate cAMP, therefore, currently being intensively investigated as potential inducers of mitochondrial biogenesis through the deacetylation-mediated activation of PGC-1α (Chowanadisai et al., 2010; Park et al., 2012). The polyphenols curcumin are components of *Curcuma longa L*, a compound with medicinal properties, and turmeric, a popular culinary spice in both vegetarian and non-vegetarian foods. A previous study had shown that the anti-oxidant activities of curcumin were more potent than those of another polyphenol, resveratrol (Aftab et al., 2010). Furthermore, the long-term effects of dietary curcumin on various markers of mitochondrial biogenesis have

also been investigated. Five-month dietary supplementation of curcumin has been shown to up-regulate PGC-1α protein expression in senescence-accelerated mouse-prone 8 (SAMP8), a fast-aging mouse strain, thereby improving mitochondrial membrane potential (MMP) and ATP levels and restoring mitochondrial fusion in the brain (Eckert et al., 2013). However, the effects of curcumin on the skeletal muscle and its underlying mechanisms in the regulation of mitochondrial biogenesis are yet to be elucidated.

In the present study, we have investigated the mechanisms by which curcumin affects mitochondrial biogenesis in rats. We predicted that treatment with curcumin will have effects on the induction of mitochondrial biogenesis, and combined with endurance training (eTR), curcumin treatment addictive or synergistically to enhance eTR-induced mitochondrial biogenesis. The primary purpose of the present study was to determine the effects of curcumin treatment combined with 24 days of eTR on regulation of mitochondrial biogenesis in skeletal muscles (m. gastrocnemius [Gas] and m. soleus [Sol]). In the present study, we provide the evidence of the efficacy of curcumin treatment and eTR on increasing mitochondrial biogenesis in skeletal muscle. We hypothesized that there may be potentially be an addictive or synergistic effect on mitochondrial biogenesis.

II. Previous Research

1. Mitochondrial Structure and Function

Mitochondria are abundantly present in mammalian cells. Their fraction variation from tissue to tissue, ranging from < 1% (volume) in white blood cells to 35% in heart muscle cells (Wiesner, 1997). The mitochondria in skeletal muscle are divided into two morphologically different sub-fractions that are regionally distinct in the muscle fiber. These are subsarcolemmal (SS) mitochondria that lie directly beneath the sarcolemmal membrane and the intermyofibrillar (IMF) mitochondria that are located in close contact with the myofibrils. The mitochondria are equipped with double membranes, creating the intermembrane space between the outer and inner membranes as well as the inner matrix compartment, where most of the metabolic processes take place. The inner membrane is highly folded, forming so-called cristae, to accommodate its large surface area. Embedded in the inner mitochondrial membrane contains the five complexes that make up the respiratory chain where oxidative phosphorylation takes place.

Mitochondria are the main site of ATP synthesis, using the free energy of the oxidation of metabolic fuels with oxygen. The matrix space which containing the enzymes of the citrate cycle and beta-oxidation, enclosed by an inner membrane containing the 4 complexes of the electron transport chain, ATP synthase and specific carriers for metabolites. Mitochondria also have a relatively permeable outer membrane and an intermembrane space. ATP

synthesis (oxidative phosphorylation) is critically dependent on the structural integrity of the mitochondrion. Electrons from substrate oxidations feed into the electron transport chain at complex I or complex II, and then successively flow to complex III, complex IV and finally to oxygen. Complexes I, III and IV are redox pumps and electron transport causes extrusion of protons from the matrix generating an electrochemical proton gradient (proton motive force) across the inner membrane. Protons return to the matrix through ATP synthase driving the synthesis of ATP. The stoichiometry of proton extrusion and the yield of ATP are still uncertain. Mitochondria have genetic continuity and are inherited maternally. They possess a small amount of DNA which codes for some, but not all, of the subunits of complexes I, III, IV of ATP synthase. mtDNA also codes for mitochondrial ribosomal and messenger RNAs involved in the synthesis of mitochondrially coded subunits. All other mitochondrial peptides are synthesised on cytosolic ribosomes and are imported and targeted to their specific intramitochondrial locations, often after proteolytic removal of leader sequences (Sherratt, 1991).

2. Mitochondrial Biogenesis

A. Definitions

Mitochondrial biogenesis can be defined as the growth and division of pre-existing mitochondria. Due to their ancient bacterial origin, mitochondria have their own genome and a capacity for autoreplication. Mitochondrial proteins are encoded by the nuclear and the mitochondrial genomes. The double-strand

circular mitochondrial DNA (mtDNA) is ~16.5 kb in vertebrates and contains 37 genes encoding 13 subunits of the electron transport chain (ETC) complexes I, II, III, IV, and V, 22 transfer RNAs, and 2 ribosomal RNAs necessary for the translation. Correct mitochondrial biogenesis relies on the spatiotemporally coordinated synthesis and import of 1000 proteins encoded by the nuclear genome, of which some are assembled with proteins encoded by mitochondrial DNA within newly synthesized phospholipid membranes of the inner and outer mitochondrial membranes. In addition, mitochondrial DNA replication and mitochondrial fusion and fission mechanisms must also be coordinated. All of these processes have to be tightly regulated in order to meet the tissue requirements. Mitochondrial biogenesis is triggered by environmental stresses such as exercise, cold exposure, caloric restriction and oxidative stress, cell division and renewal, and differentiation. The biogenesis of mitochondria is accompanied by variations in mitochondrial size, number, and mass. The discovery that alterations in mitochondrial biogenesis contribute to cardiac pathologies such as the hypertrophied or failing heart have increased the interest of the scientific community in this process and its regulation.

B. Protein Import into Mitochondria

Because the majority of mitochondrial proteins are encoded in the nucleus, a mechanism for the targeting, import, and correct assembly exists to ensure proper mitochondrial function and shape (Baker et al., 2007; Hood et al., 2003). Following activation of the nuclear genome, mRNAs are translated in the cytosol to precursor

proteins having signals for targeting to specific mitochondrial compartments. These proteins are escorted by molecular chaperones, unfolded, and imported into mitochondria via the translocase of the outer membrane complex (TOM). After transfer across the outer membrane, certain precursors are directed through the import machinery of the inner membrane complex (TIM) into the mitochondrial matrix in a membrane potential-dependent manner. Subsequently, these precursors are cleaved of their import sequences and are refolded by intramitochondrial proteins. A majority of mitochondrial protein precursors, however, do not contain typical N-terminal targeting signals but instead contain targeting information withintheir mature sequences. Other mechanisms ensure proper assembly and processing of the different subunits in the mitochondrial complexes of the respiratory chain at the inner membrane or in the matrix. These processes are an integral part of mitochondrial biogenesis.

C. Mitochondrial Fusion and Fission

Mitochondria in the cells of most tissues are tubular, and dynamic changes in morphology are driven by fission,fusion, and translocation (Bereiter, 1990). The ability to undergo fission/fusion enables mitochondria to divide and helps ensure proper organization of the mitochondrial network during biogenesis. Mitochondrial fission is driven by dynamin-related proteins 1 and optic atrophy 1 (DRP1and OPA1), while mitochondrial fusion is controlled by mitofusins (Mfn1 and 2). Mitofusins are highly expressed in heart and skeletal muscle, and their expression is induced during myogenesis and physical exercise (Bach et al.,

2003; Soriano et al., 2006). In addition to the control of the mitochondrial network, Mfn2 also stimulates the mitochondrial oxidation of substrates, cell respiration, and mitochondrial membrane potential, suggesting that this protein may play an important role in mitochondrial metabolism, and as a consequence, in energy balance. OPA1, by contrast, is involved in the remodelling of cristae. Mfn and DRP1 expression increases in parallel with mitochondrial content and exercise capacity in human skeletal muscle (Garnier et al., 2005) , suggesting that fusion/fission processes are an integral part of mitochondrial biogenesis.

D. Factor Regulating Mitochondria Biogenesis

a. DNA-Binding Transcription Factors Nuclear Respiratory Factor-1, Regulator of OXPHOS and mtDNA Replication/Transcription Factors

NRF-1 was identified as a transcription factor binding to a conserved regulatory site of the *cytochrome c* promoter. NRF-1 binding sites are evolutionarily conserved in the proximal promoters of many mitochondrial genes (Gollnick et al., 1972). Accordingly, NRF-1 activates the expression of OXPHOS (oxidative phosphorylation) components, mitochondrial transporters, and mitochondrial ribosomal proteins. In addition, NRF-1 regulates expression of *Tfam*, *Tfb1m*, and *Tfb2m* and thereby coordinates the increased expression of nuclear mitochondrial genes with increases in mtDNA replication and expression (Gollnick et al., 1972). NRF-1 may also affect expression of mitochondrial and metabolic genes via indirect mechanisms, e.g., by inducing expression of the transcription factor MEF2A (Myocyte-specific enhancer factor 2A), which

activates COX (cytochrome c oxidase) genes, GLU4, and PGC-1α (Baar et al., 2002). Silencing of NRF-1 leads to a significant suppression of mitochondrial target genes, suggesting that endogenous NRF-1 is constitutively active and important for the basal expression of mitochondrial targets ((Meirhaeghe et al., 2003; Ventura-Clapier et al., 2008). Nevertheless, NRF-1 activity can also be regulated by phosphorylation and/or interactions with PGC-1α, PGC-1β, PRC (PGC-related coactivator), and cyclin D1. Phosphorylation of NRF-1 occurs upon exposure of quiescent fibroblasts to serum (which correlates with induction of Cycs) and exposure of hepatoma cells to oxidants (which leads to an NRF-1-dependent induction of Tfam). Depending on the context, phosphorylation affects NRF-1 translocation to the nucleus, DNA binding, and/or transcriptional activity (Gollnick et al., 1972). Physical interactions of the PGC-1 family members with NRF-1 enhance NRF-1-dependent gene expression (Bergeron et al., 2001; Puigserver et al., 1998). Many signals known to induce mitochondrial biogenesis or respiratory function also induce NRF-1 expression, suggesting that NRF-1 is part of the energy-sensing pathway in mammalian cells. In vivo, NRF-1 expression in muscle is induced by exercise in rat (Narkar et al., 2008). Finally, NRF-1 expression was induced in the muscle of rats fed with a creatine analog that activates AMP-activated protein kinase (AMPK) and induces adaptations similar to those induced by exercise training (Nisoli et al., 2003). All together, two major signals have emerged as regulators of NRF-1 expression: increases in Ca^{2+} and activation of AMPK. Whether these signals regulate NRF-1 activity directly, and not simply NRF-1 expression, is not yet known. In support of a role

for NRF-1 as a critical transcription factor for expression of mitochondrial genes, NRF-1 null animals show early embryonic lethality, and NRF-$1^{-/-}$ blastocysts have reduced mtDNA content and mitochondrial membrane potential (Rodgers et al., 2005). Although NRF-1 seems necessary for mitochondrial biogenesis, its expression alone is not sufficient to drive this program. Transgenic overexpression of NRF-1 in muscle increases expression of select NRF-1 targets but does not enhance respiratory capacity, suggesting that activation of parallel transcription pathways must complement NRF-1 during exercise-induced muscle mitochondrial biogenesis (Lagouge et al., 2006).

b. Mitochondrial Transcription Factor A (TFAM)

The first mitochondrial transcription factor described was TFAM (Fisher et al., 1985), a nuclear encoded protein that is translocated to the mitochondria where it exerts its effects by binding to mtDNA. TFAM is a member of the high-mobility group (HMG) of proteins. It has the ability to bind, unwind and bend DNA without sequence specificity, but show higher affinity for LSP and HSP. C-terminal deletion of TFAM resulted in loss of specific DNA binding and transcription activation of the protein. The vital role of TFAM during development and maintenance of the organism is shown by the lethality of homozygous TFAM knock-out in both mice (Larsson et al., 1998). Homozygous TFAM knock-out mice die between embryonic days 8.5-10.5. The mutant embryos lack mtDNA, and display severe respiratory chain deficiency with massive accumulation of morphologically abnormal mitochondria, as well as

delayed neutral development and defective heart. The biological regulatory function is further demonstrated by that TFAM level varies with the level of mtDNA in human cell (Larsson et al., 1994). Completely about mitochondrial biogenesis mechanism included factors which involve in regulating mitochondrial biogenesis has showed in Figure II-1.

3. Transcription Cascade of Mitochondrial Biogenesis

A. PGC-1α (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha)

Mitochondria are composed of 1,500 proteins, which are encoded by both the nuclear and mitochondrial DNA (mtDNA). The mtDNA encodes only 13 of these polypeptides, which are subunits of the mitochondrial ETC. It also encodes for two rRNA genes and 22 tRNA genes required for mitochondrial protein synthesis. All other mitochondrial proteins are encoded by the nuclear DNA. Consequently, the process of mitochondrial biogenesis requires coordination between these two genomes. This coordination is regulated primarily by the PGC-1 family of transcriptional coactivators, which work by activating transcription factors. However, they do not interact directly with DNA. As described below, they bind to several different transcription factors that bind DNA. PGC-1α coactivators also interact with a histone acetyltransferase complex that consists of cAMP response element binding protein/p300 that facilitates chromatin remodeling and also with the thyroid hormone receptor-

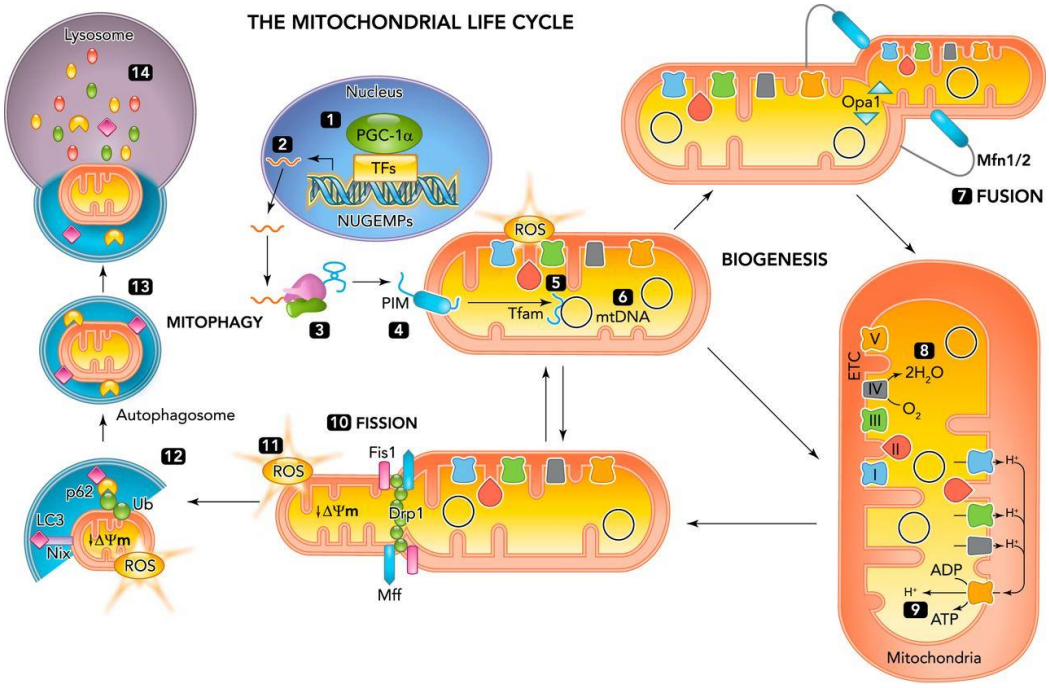


Figure II-1. The mitochondrial life cycle (Carter et al., 2015).
Under steady-state conditions, mitochondrial turnover occurs through a combination of organelle synthesis and degradation. Binding of transcriptional coactivators, such as PGC-1α, to transcription factors (TFs) results in the expression of nuclear genes encoding mitochondrial proteins (NUGEMPs; *1*) and production of multiple mRNAs (*2*). These mRNAs are exported from the nucleus and translated into protein in the cytosol (*3*). With the assistance of chaperones, proteins destined for the mitochondria will be directed to the protein import machinery (PIM) for import into the organelle (*4*). Once inside, the proteins are directed to the appropriate mitochondrial compartment, and the organelle can expand to contain more ETC machinery. Tfam is a matrix-destined protein that binds mtDNA (*5*) to regulate transcription and mtDNA replication (*6*). Two adjacent organelles can be tethered and fused together through the fusion proteins (Mfn1/2 and Opa1) as an additional mechanism for organelle expansion (*7*). Healthy mitochondria consume oxygen (*8*) and produce ATP (*9*) in the electron transport chain (ETC), in accordance with the cellular demand. When a portion of the mitochondrial network becomes damaged, fission proteins (Fis1, Mff, and Drp1) can be recruited to the dysfunctional site to cleave off the damaged portion (*10*). Typically, dysfunctional mitochondria are recognized through an increase in ROS emission and lower membrane potential (*11*). Once the damaged mitochondrion is separated from the network, it can be flagged for mitophagic degradation by ubiquitination of outer membrane proteins and binding of p62, LC3II, and NIX (*12*). Lipidated LC3II will initiate autophagosome formation to surround the organelle (*12*). Once fully encapsulated, the autophagosome (*13*) is directed to the lysosome. Fusion of the autophagosome with the lysosome results in the degradation of the organelle by proteolytic enzymes to its basic constituents (*14*).

associated protein/vitamin D receptor-interacting protein (TRAP/DRIP) mediator complex in order to activate DNA transcription. In addition to regulating mitochondrial biogenesis, the PGC-1 α coactivators are involved in modulating other metabolic pathways including fatty acid oxidation (FAO), lipogenesis, gluconeogenesis, and thermogenesis. The PGC-1α family initiates mitochondrial biogenesis by activating transcription factors that regulate the expression of nuclear DNA encoded mitochondrial proteins (Ventura et al., 2008). These transcription factors include the nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2) and the estrogen related receptor alpha (ERRα). The activation of these transcription factors increases the expression of many mitochondrial proteins, including the mitochondrial transcription factor A (Tfam) (Bergeron et al., 2001) .

Tfam is essential for mtDNA replication, transcription, and maintenance. Therefore, by regulating Tfam levels, the PGC-1α coactivators are able to influence the expression of proteins encoded by the mtDNA. The proteins encoded by the nuclear DNA are imported into the mitochondria where some will combine with proteins encoded by the mtDNA to form the multi subunit complexes of the mitochondrial OXPHOS system. Therefore, the PGC-1α family regulates mitochondrial biogenesis by coordinating the expression of mitochondrial proteins encoded by both the nuclear and mitochondrial genomes (Figure II-2). Although PGC-1α acts mainly in the nucleus more recently, Aquilano et al. (2010) suggested that PGC-1α and the NAD dependent

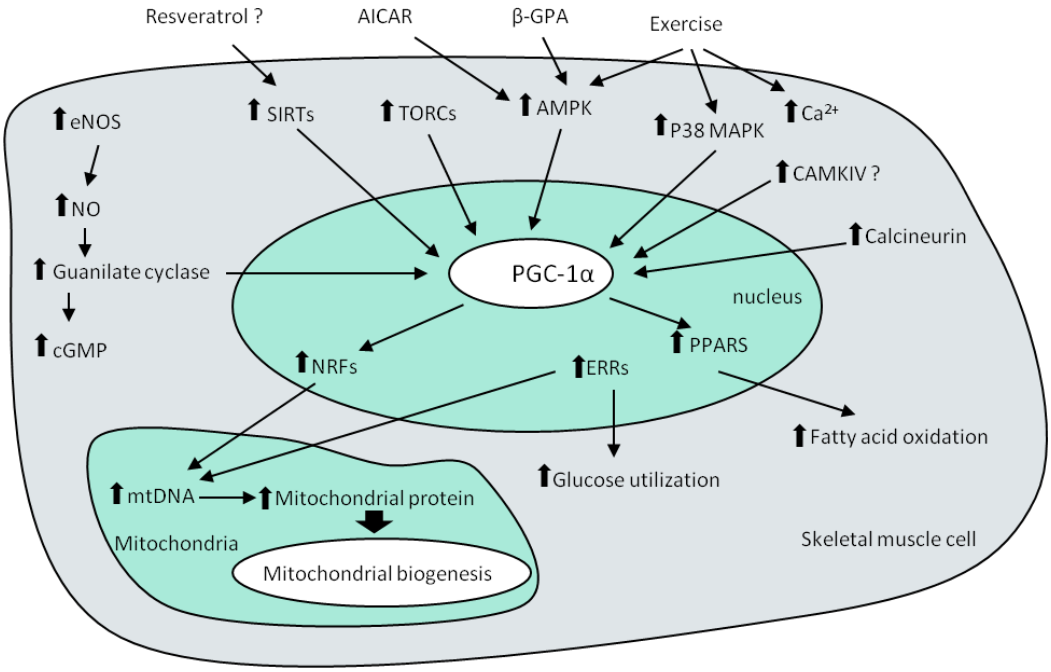


Figure II-2. PGC-1α coactivators are master regulators of mitochondrial biogenesis (Jornayvaz et al., 2010).
PGC-1α coactivators regulate mitochondrial biogenesis by coordinating the expression of mitochondrial proteins encoded by both the nuclear and mitochondrial genome. To accomplish this, they activate transcription factors such as NRF-1/2 and ERRa, thereby increasing the expression of nuclear DNA-encoded mitochondrial proteins. These mitochondrial proteins are then imported into the mitochondria. One such protein is TFAM, which when upregulated, leads to increased mtDNA replication and increased expression of mtDNA-encoded proteins. These processes initiate an increase in mitochondrial biogenesis.

protein deacetylase sirtuin 1 (SIRT1), another pivotal regulator of mitochondrial biogenesis, are located in the mitochondria. This is an interesting model as before this study, these proteins were thought to function only in the nucleus. Using fluorescent microscopy, the authors showed that PGC-1α and SIRT1 did not only colocalize with the nuclear Hoechst stain but also with cytochrome c, a mitochondrial protein used to label the mitochondrial network (Aquilano et al., 2010; Dillon et al., 2012). In addition, they showed Tfam interacts with PGC-1α and SIRT1 in the mitochondria to form multiprotein complexes (Aquilano et al., 2010). Moreover, they found that the mtDNA D-loop region was present in crosslinked mitochondria immunoprecipitated with anti-PGC-1α and that PGC-1α and SIRT1 were present in nucleoid (a poorly defined structure containing mtDNA and proteins) enriched mitochondrial fractions (Aquilano et al., 2010).

Studies performed in mammalian cells and mouse models have highlighted the importance of the PGC-1 family in the regulation of mitochondrial biogenesis. The overexpression of PGC-1α in C2C12 muscle cells, cardiac myocytes, and primary rat neurons was shown to increase mitochondrial density, the expression of nuclear and mitochondrial genes and mitochondrial function (Lehman et al., 2000; St-Pierre et al., 2003). Also, increased PGC-1α expression in mouse heart and skeletal muscle triggers an increase in mitochondrial biogenesis (Russell et al., 2004; Wenz et al., 2009). Accordingly, the ablation of PGC-1α in mouse skeletal muscle and heart leads to decreased expression of most nuclear and mitochondria encoded genes, decreased mitochondrial enzyme activity and mitochondria content (Adhihetty et al., 2009). These studies

demonstrate that PGC-1α play pivotal roles in regulating mitochondrial biogenesis and function.

B. AMPK (5' Adenosine Monophosphate-Activated Protein Kinase)

AMPK is a major regulator of mitochondrial biogenesis which regulates intracellular energy metabolism in response to acute energy crises (Hardie, 2007). β-GPA (β-guanadinopropionic acid) is a creatine analogue acting as a chronic pharmacological activator of AMPK. Indeed, β-GPA mimics exercise training and leads to reductions in the intramuscular ATP/AMP ratio and phosphocreatine concentrations, which in turn activates skeletal muscle AMPK. Rats fed for 8 weeks with β-GPA had a chronic skeletal muscle AMPK activation, which resulted in increases in NRF-1-binding activity, δ-ALAS mRNA expression, cytochrome *c* protein expression and mitochondrial content, thus demonstrating that AMPK activation promotes mitochondrial biogenesis through PGC-1α and the NRFs (Bergeron et al., 2001). To further examine the role of AMPK in mitochondrial biogenesis, transgenic mice overexpressing a DN (dominant-negative) mutant of AMPK in muscle (DN-AMPK) were treated with β-GPA. This treatment had no effect on AMPK activity or mitochondrial content in DN-AMPK mice, but induced an activation of muscle AMPK and mitochondrial biogenesis in wild-type mice. Furthermore, this AMPK inactivation abrogated the β-GPA-induced expression of PGC-1α and CaMKIV (calcium/calmodulin-dependent protein kinase IV) (Zong et al., 2002).

Pharmacological activation of skeletal muscle AMPK enhances mitochondrial biogenesis, an effect shown to be dependent largely on expression of the AMPK α2 subunit (Jorgensen et al., 2007) and peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) (Leick et al., 2009). The overexpression of constitutively active skeletal muscle AMPK also increases PGC-1α and mitochondrial function, further supporting a critical role for this pathway in regulating mitochondrial biogenesis (Garcia-Roves et al., 2008). Consistent with these findings, mice lacking AMPK β (O'Neill et al., 2011) or liver kinase B1 (LKB-1) (Jeppesen et al., 2013; Tanner et al., 2013) have reduced muscle mitochondrial contents. Many studies have investigated the mechanisms by which AMPK may regulate mitochondrial biogenesis, and this has been shown to involve both direct phosphorylation and acetylation (via sirtuin 1) of PGC-1α (O'Neill et al., 2013). However, with exercise, mice lacking PGC-1α (Adhihetty et al., 2009) or sirtuin 1 have normal increases in mitochondrial biogenesis (Philp et al., 2011). Interestingly, in LKB-1 muscle null mice, exercise training does not increase components of the mitochondrial electron transport chain (Tanner et al., 2013). Taken together, these data demonstrate that by sensing the energy status of the muscle cell, AMPK is a key regulator of mitochondrial biogenesis.

C. SIRT1 (Sirtuin 1)

Evidence was shown that PGC-1α was in fact a deacetylation target of SIRT1 and that acetylation regulated PGC-1α activity (Sack et al., 2012; Nemoto

et al., 2005; Rodgers et al., 2005). There are at least 13 lysine residues on PGC-1α that appear to be reversibly acetylated (Rodgers et al., 2005). SIRT1 appears to be the predominant in vitro and in vivo regulator of PGC-1α deacetylation. In particular, in skeletal muscle, fasting was shown to lead to a SIRT1-dependent deacetylation of PGC-1α, and this deacetylation appeared to be required for PGC-1α-dependent gene expression, including gene products required for effective mitochondrial biogenesis (Gerhart-Hines et al., 2007). The above studies suggest that mitochondrial biogenesis might be regulated by tissue energetic status and that the sirtuins would represent important energy sensors in this homeostatic loop. Indeed, the notion that PGC-1α acetylation and function, and by extension mitochondrial activity, are regulated in a nutrient-dependent fashion by SIRT1 is appealing. Nonetheless, the concept that SIRT1 is in turn responding to nutrient-sensitive changes in basal NAD levels, although often invoked, has until recently had little experimental support. The difficulty in proving the supposition is that measurement and manipulation of NAD levels in various subcellular compartments is experimentally challenging. One recent approach is to carefully examine mice with a deletion of a major NAD-consuming enzyme, poly (ADP-ribose) polymerase-1 (PARP-1). These mice appear to have elevated NAD levels along with increased SIRT1 activity (Bai et al., 2011). Another recent report suggested that adiponectin, a secreted adipokine, could regulate intracellular NAD levels. Again, in these studies the addition of adiponectin to cells appeared to increase mitochondrial content through a SIRT1- and PGC-1α-dependent pathway. These results might be particularly important

because metabolic diseases are often associated with low adiponectin levels (Hotta et al., 2000), as well as with mitochondrial dysfunction (Petersen et al., 2004), although the link between these two observations were previously unknown. The generation of new mitochondria through a SIRT1 / PGC-1α-regulated pathway is complemented by another important connection between SIRT1 and the mitochondria. In particular, it would appear that SIRT1 is an important regulator of removing damaged mitochondria through the process of autophagy (Lee et al., 2008). Evidence suggests that SIRT1 can stimulate autophagy and that $SIRT1^{-/-}$ tissues appear to accumulate abnormal-appearing mitochondria, consistent with what is seen in autophagy-deficient tissues (Lee et al., 2008).

The molecular basis for how SIRT1 stimulates autophagy is not clear. There is evidence that key molecules required for autophagy including Atg5 and Atg7 are direct targets of sirtuin-dependent deacetylation (Lee et al., 2008). In addition, the FoxO family of transcription factors, known targets of SIRT1 deacetylation as well as regulators of autophagy, has also been implicated (Hariharan et al., 2010). Nonetheless, although details remain to be elucidated, the notion that SIRT1 can regulate both the creation of new mitochondria as well as the removal of old mitochondria suggests a role for sirtuins in overall mitochondrial flux and in the maintenance of what may be viewed as "youthful" mitochondria in the cell. There is also evidence that a recently described extracellular, circulating form of the enzyme nicotinamide phosphoribosyltransferase (Nampt) may be an important in vivo mediator of

insulin secretion (Revollo et al., 2007). The Nampt enzyme is a key enzyme in NAD biosynthesis and is responsible for the conversion of nicotinamide to nicotinamide mononucleotide, a metabolite that can in turn be converted directly to NAD. Again, these observations highlight the potential function of SIRT1 in linking metabolites such as NAD to the maintenance of overall metabolic homeostasis.

D. cAMP (*Cyclic Adenosine Monophosphate)*

Cyclic AMP is generated from ATP via adenylyl cyclases (ACs) and degraded via phosphodiesterases (PDEs). The cAMP generated by adenylyl cyclases is tightly regulated by phosphodiesterases (PDE), which catabolize cAMP into 5′ AMP. There are 11 known PDE families; some are specific for cAMP (PDEs 4, 7, 8), others for cGMP (PDEs 5, 6, 9), whereas the remainder (PDEs 1–3, 10, 11) catabolize both cAMP and cGMP. Each of the PDE families includes multiple isoforms with distinct enzymatic characteristics, modes of regulation, expression patterns, and distribution throughout the cell. Cyclic AMP effector molecules contribute to the complexity and specificity of cAMP signaling. PKA, a well studied cAMP downstream effector, is a tetrameric enzyme consisting of two catalytic domains (C) and two regulatory domains (R). In mammals, there are three known isoforms of catalytic subunits (Cα, Cβ, Cγ) and four isoforms of regulatory subunits (RIα, RIβ, RIIα, RIIβ). cAMP binding to R releases active C subunits, which phosphorylate key substrates (Kim et al.,

2005). A structurally diverse group of proteins called A kinase anchoring proteins (AKAPs) direct PKA to distinct subcellular sites (Diviani et al., 2011).

E. CREB (cAMP Response Element Binding Protein)

The signal transduction pathway leading to expression of nuclear genes encoding mitochondrial proteins is believed to be regulated by CREB-dependent transcription of PGC-1α, a co-activator of these genes and master regulator of mitochondrial biogenesis (Conkright et al., 2003). An important mechanism of CREB activation of PGC-1α in this pathway is the activation and nuclear translocation of CRTC, a family of CREB co-activators that are known to respond to metabolic stress mediated through cAMP- dependent protein kinase (PKA) or AMP-activated protein kinase (AMPK) (Wang et al., 2009). The cAMP response element-binding protein (CREB) is a ubiquitous transcription factor that regulates the transcription of cAMP response element-regulated genes. It belongs to the CREB/ATF1 (Cyclic AMP-dependent transcription factor ATF-1) family of cAMP/Ca^{2+} responsive transcription factors. PKA phosphorylation activates CREB. Nuclear CREB regulates PGC-1α, the master regulator of mitochondrial biogenesis (Chowanadisai et al., 2010). Blocking CREB or PGC-1α in mouse hepatocytes inhibited mitochondrial biogenesis induced by pyrroloquinoline quinone (PQQ), a redox cofactor and antioxidant compound. In addition to its nuclear roles, CREB is also found bound to the mitochondrial DNA (Cammarota et al., 1999). In brain, mitochondrial CREB regulates mitochondrial gene expression and neuronal survival (Lee et al., 2005). CREB is

thought to be transported to the mitochondria via chaperone molecules, such as Hsp70 (70 kilodalton heat shock proteins). CREB uptake was promoted by the mitochondrial membrane potential and by TOM complex, responsible for the translocation of the proteins from the cytoplasm into the mitochondria. Once inside mitochondria, CREB interacts with cAMP response elements (CREs) in the mitochondrial DNA, thereby regulating expression of mitochondrial components of the electron transport chain. It appears to bind directly to the D-loop of mitochondrial DNA. Mitochondrial CREB phosphorylation was shown to regulate expression of mitochondrial DNA-encoded proteins, such as ND1 (NADH dehydrogenase 1), ND6 (NADH dehydrogenase 6), and COX-III (cytochrome c oxidase subunit III / ATP6 Adenosine Triphosphate Synthase Subunit 6). Based on experiments with either wild-type or Ser133 mutant mitochondrially targeted CREB, It was proposed that CREB can be phosphorylated by PKA within mitochondria (Ryu et al., 2005).

F. LKB-1 (Liver Kinase B1)

Liver kinase B1 (LKB-1) was discovered only 12 years ago, as a serine–threonine kinase that is mutated in Peutz-Jeghers Syndrome. Initially, studies to identify the function of LKB-1 were difficult, as its sequence gave few clues as to its activity. Overall it had little similarity to other protein kinases. However, studies in cell lines including HeLa S3 and G361 melanoma cells, which lack endogenous LKB-1, provided insights into LKB-1's role as a tumor suppressor, including early evidence of its role in stress and damage responses. More

recently, LKB-1 has been appreciated to be a signaling protein, integrating cellular energy sensing with growth and proliferation, functioning both in the nucleus and the cytoplasm. It is ubiquitously expressed throughout the body, and functions as a heterotrimer with STRAD (sterile-20-related adaptor) and MO25 (mouse protein-25) in cells. LKB-1 is post-translationally modified by kinases in several signaling pathways including cAMP–PKA (Alexander and Walker., 2011). Despite the fact that it is constitutively active in cells, it has also been noted that LKB-1 catalytic activity is enhanced when STRAD and MO25 are present in the complex.

AMP-activated protein kinase (AMPK) is one of the best characterized substrates of LKB-1. LKB-1 has now been shown to be one of the primary kinases that phosphorylates AMPK. AMPK activation by agonists such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), metformin or phenformin or in response to stress is absent in LKB-1-deficient cells (Hawley et al., 2003), identifying LKB-1 as an obligatory AMPK kinase in this setting. Activation of AMPK by AICAR occurs via mono-phosphorylation of AICAR to its active form, ZMP which can mimic the effect of AMP on AMPK, i.e., allosteric activation and enhancement of phosphorylation by upstream kinase(s). In addition to LKB-1, the calmodulin-dependent protein kinases CaMKKα and CaMKKβ also function as kinases for Thr 172 of AMPK. This activation pathway is calcium-responsive, does not require an increase in AMP, and is thought to be particularly important for endocrine hormone regulation, for example adiponectin activation of AMPK in vascular endothelial cells. CaM-

kinases are most highly expressed in neural tissue, where they play a role in activating AMPK in response to neuronal depolarization caused by K^+-increases and Ca^{2+} influx.

4. Exercise Induces Mitochondrial Biogenesis

Endurance exercise has a number of health benefits, including improvements in muscle metabolism, cardiovascular function, and increased exercise tolerance. The increase in endurance is a result of greater oxygen delivery and extraction by the exercising muscle. Oxygen extraction is a result of an improved capillary-to-fiber ratio, as well as a higher mitochondrial content within muscle. The increase in mitochondrial content is a well-established adaptation within the exercised muscle, but the molecular mechanisms underlying this change in muscle phenotype still remains to be clarified. This increase in mitochondrial content which occurs as a result of regular exercise is referred to as mitochondrial biogenesis.

A. The Role of PGC-1α as a Regulator of Exercise Induces Mitochondrial Biogenesis

PPARγ coactivator-1α (PGC-1α) is the 'master regulator' of mitochondrial biogenesis because of its ability to induce mitochondrial biogenesis in a variety of experimental models. For example, the overexpression of PGC-1α in skeletal muscle of transgenic mice is sufficient to coordinate a host of muscle adaptations reminiscent of endurance exercise training, including increased mitochondrial

content, increased proportion of Type I muscle fibers, and a corresponding increase in muscle fatigue resistance. PGC-1α binds to and coactivates DNA-binding transcription factors, thus augmenting their activity. The primary targets of PGC-1α are the nuclear respiratory factors, NRF-1 and NRF-2. PGC-1α increases the expression of both these transcription factors, and coactivates NRF-1-mediated transcription. NRF-1 and/or NRF-2 binding sites are found in the promoter regions of several nuclear genes encoding mitochondrial proteins, including cytochrome *c*, components electron transport chain complexes, mitochondrial import proteins, heme biosynthesis proteins, and the mitochondrial transcription factors Tfam. Thus, working through NRF-1, PGC-1α coordinates the bi-genomic regulation of mitochondrial biogenesis. The expression of PGC-1α is regulated by altered patterns of physical activity. In response to a single bout of exercise, PGC-1α mRNA and protein are significantly elevated in mice and rats, and humans (Hood and Saleem, 2007). Increase in gene expression is evident as early as 2 h post-exercise. Although exercise elicits numerous systemic changes in the body, the exercise-induced PGC-1α upregulation appears to be independent of these factors. Evidence for this comes from the fact that contractile activity alone (i.e. no humoral influence) is sufficient to increase PGC-1α expression. Together, these studies point to contractile activity as the main stimulus for exercise-induced PGC-1α upregulation.

B. *Calcium and AMP Kinase Signaling to Exercise Increase Mitochondrial Biogenesis*

a. *Ca^{2+} Signaling and Exercise*

In skeletal muscle, Ca^{2+} acts as an essential regulatory and signaling molecule. Gene expression of respiratory proteins in skeletal muscle has been linked to intracellular Ca^{2+} signaling mediated by Ca^{2+}-dependent regulatory enzymes, namely calcineurin, Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and protein kinase C (PKC). The strongest evidence favoring Ca^{2+}-mediated alterations in mitochondrial biogenesis come from studies with CaMKIV isoform overexpression. Transgenic mice possessing a constitutively active CaMKIV exhibited increased muscle PGC-1α expression, mitochondrial biogenesis, and an up-regulation of mitochondrial gene expression (Wu et al., 2002). CaMK increases PGC-1α promoter activity through the activation of cAMP response element binding protein (CREB) (Handschin et al., 2003). Electrical stimulation of skeletal muscle in mice activates the PGC-1α promoter, and this effect is abolished when CREB is mutated (Akimoto et al., 2004). These studies point to CREB in altering PGC-1α transcription in response to multiple exercise-induced signals. Surprisingly, the response of CaMKIV knockout mice to endurance training, including an upregulation of PGC-1α, is indistinguishable from that of wildtype (WT) mice. Thus, other CaMK isoforms, more abundantly expressed in muscle, may be important for the exercise-induced upregulation of PGC-1α (Chin, 1985).

b. AMPK Activation with Exercise

5′-AMP activated protein kinase (AMPK) is an energy-sensing enzyme that responds to cellular conditions associated with energy depletion. AMPK has been shown to be activated by exercise in both animals and humans. The enzyme is a heterotrimer that consists of a catalytic α subunit and two regulatory subunits, β and γ. The signaling cascade that involves AMPK phosphorylation and activation is the result of a reduction in the ATP/ADP ratio, and a concurrent elevation in AMP due to myokinase activity, such as that which occurs during contractile activity. AMPK is allosterically activated by AMP up to 10-fold (Carling et al., 1987) and is antagonized by ATP which competes for the same binding site (Corton et al., 1995). AMPK can also be phosphorylated and activated by one or more upstream protein kinases that create a more than 100-fold activation. Activation of AMPK also occurs with 5-aminoimidazole-4-carboxamide riboside (AICAR) treatment. AICAR is taken up by cells and phosphorylated by cellular adenosine kinase to ZMP, an analog of AMP. Pharmacological activation of AMPK by AICAR increases PGC-1α mRNA (Terada et al., 2002) and protein (Irrcher et al., 2003). This upregulation of PGC-1α transcription and translation is accompanied by the increased DNA binding activity of NRF-1. As noted above, NRF-1 is a transcriptional regulator of proteins involved in mitochondrial biogenesis. In addition, chronic activation of AMPK using AICAR in resting rats has resulted in increases in mitochondrial enzymes such as δ-aminolevulinic synthase, cytochrome *c*, citrate synthase and malate dehydrogenase in skeletal muscle (Winder et al., 1985). Thus, AMPK

activation is another important regulator of mitochondrial biogenesis under conditions of energy deprivation in muscle cells.

c. SIRT1 Activation with Exercise

Silent information regulator 2 is an oxidized form of nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase that is required for longevity in *Caenorhabditis elegans* and *Saccharomyces cerevisiae* in response to energy restriction. In mammals, the silent information regulator 2 ortholog, SIRT1 functionally interacts and deacetylates several proteins. SIRT1 also deacetylates and functionally activates PGC-1α. SIRT1 is required for PGC-1α–induced up-regulation of mitochondrial biogenesis in skeletal muscle cells. SIRT1 plays a role in muscle gene expression in the modulation of the cytosolic NAD^+-to-NADH (reduced form of nicotinamide adenine dinucleotide) ratio. Because the cytosolic NAD^+-to-NADH ratio changes during muscle contractio, it is possible that SIRT1 contributes to skeletal muscle adaptations with endurance exercise (Suwa et al, 2008). It is therefore very likely that increased SIRT1 protein expression by endurance exercise results in elevated SIRT1 deacetylase activity as well as causing an allosteric effect of an increased cytosolic NAD^+-to-NADH ratio and then at least in part contributes to the metabolic adaptations by activating the PGC-1α in skeletal muscle (Suwa et al., 2008).

Completely how mechanism exercise / contractile activity induces mitochondrial biogenesis showed in Figure II-3.

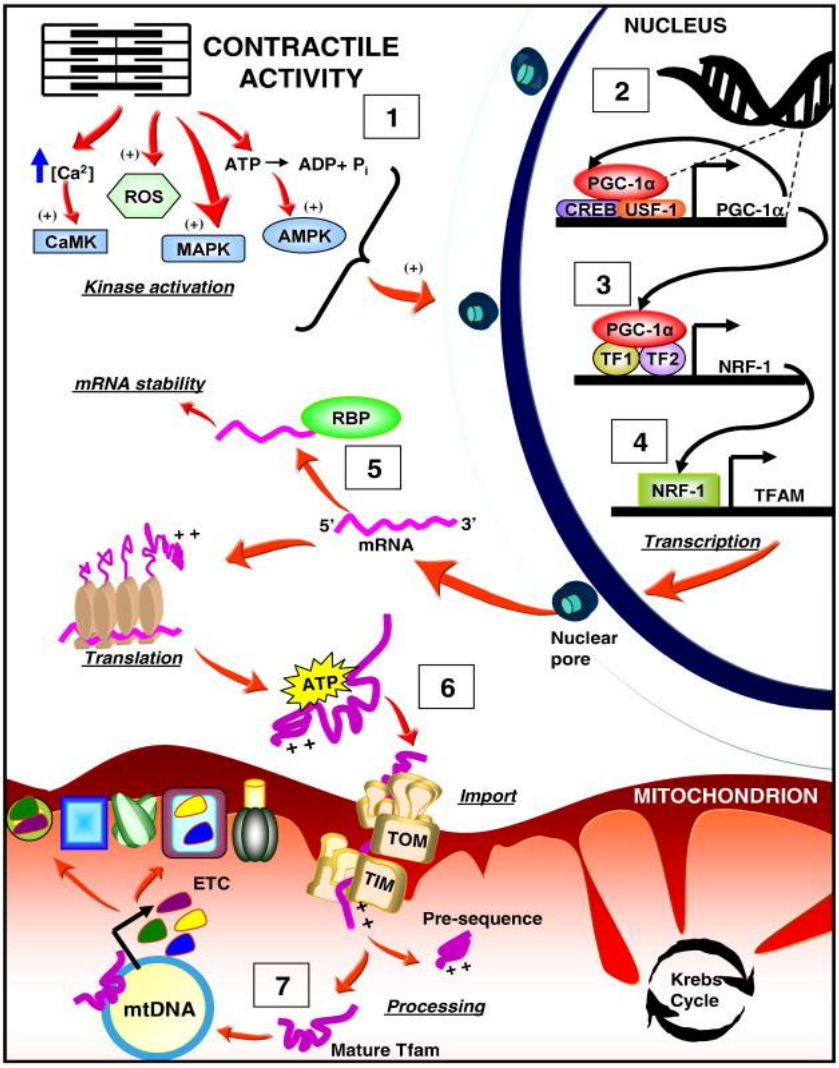


Figure II-3. Contractile activity-induced mitochondrial biogenesis (Ljubicic et al., 2010).

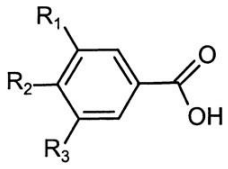
(1) A single bout of contractile activity results in the activation of multiple downstream signaling molecules and kinases. Elevations in intracellular calcium are associated with the subsequent activation of calcium/calmodulin-dependent protein kinase (CaMK). The production of reactive oxygen species (ROS) and activation of mitogen-activated protein kinases (MAPK) and AMP kinase (AMPK) also constitute the diverse array of signaling pathways that are elevated by contractile activity. (2) These multiple signaling cascades induce the binding of transcription factors to the promoter region of PPAR gamma coactivator-1α (PGC-1α) and elicit its transcription. PGC-1α, auto-regulates its own transcription and (3) binds to the promoter region of its target genes such as nuclear respiratory factor 1 and 2 (NRF-1, NRF-2) and stimulates their expression. (4) NRF-1 trans-activates nuclear genes encoding mitochondrial-destined proteins such as mitochondrial transcription factor A (TFAM). (5) These mitochondrial-destined proteins are transcribed in the nucleus and exported into the cytoplasm where the mRNA transcripts can be stabilized or destabilized by the binding of RNA-binding proteins (RBP) to the 3′ untranslated region (UTR) of the mRNA. (6) Upon translation, many mitochondrial proteins are synthesized as precursor proteins with cleavable N-terminal pre-sequences. These are imported by the protein import machinery which consists of the translocases of the outer membrane (TOM) and translocases of the inner membrane (TIM) complexes. Once imported, the precursor protein is processed to produce the mature form. (7) TFAM, a mitochondrial DNA (mtDNA) transcription factor, can induce the expression of mtDNA-encoded proteins which together with nuclear DNA-transcribed proteins, are assembled to form multisubunit complexes that become part of the electron transport chain (ETC).

5. Role of Polyphenol Induces Mitochondrial Biogenesis

A. Polyphenol

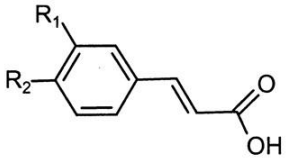
Over the past 10 years, researchers and food manufacturers have become increasingly interested in polyphenols. The chief reason for this interest is the recognition of the antioxidant properties of polyphenols, their great abundance in our diet, and their probable role in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases. Furthermore, polyphenols, which constitute the active substances found in many medicinal plants, modulate the activity of a wide range of enzymes and cell receptors (Manach et al., 2004; Middleton et al., 2000). Several thousand molecules having a polyphenol structure (ie, several hydroxyl groups on aromatic rings) have been identified in higher plants, and several hundred are found in edible plants. These molecules are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. These compounds may be classified into different groups as a function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. Distinctions are thus made between the phenolic acids, flavonoids, stilbenes, and lignans (Figure II-4). The flavonoids, which share a common structure consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C), may themselves be divided into 6 subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones,

Hydroxybenzoic acids



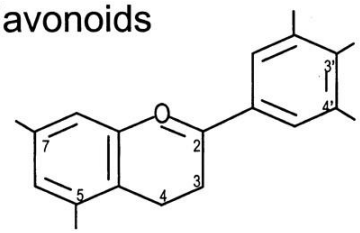
R_1 = R_2 = OH, R_3 = H : Protocatechuic acid
R_1 = R_2 = R_3 = OH : Gallic acid

Hydroxycinnamic acids

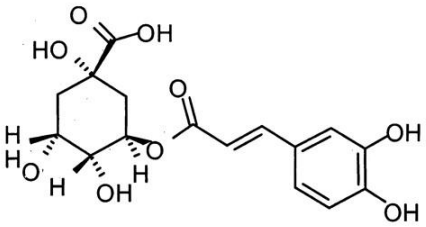


R_1 = OH : Coumaric acid
R_1 = R_2 = OH : Caffeic acid
R_1 = OCH_3, R_2 = OH : Ferulic acid

Flavonoids

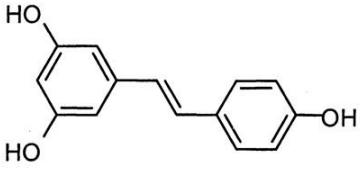


See Figure 2



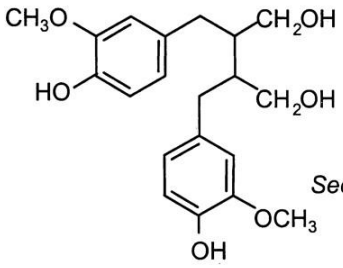
Chlorogenic acid

Stilbenes



Resveratrol

Lignans



Secoisolariciresinol

Figure II-4. Chemical structures of polyphenols (Manach et al., 2004).

anthocyanidins, and flavanols (catechins and proanthocyanidins) (Fig.II-5). In addition to this diversity, polyphenols may be associated with various carbohydrates and organic acids and with one another.

B. Mechanism Polyphenol Increase Mitochondrial Biogenesis

a. Polyphenols Increase Sirtuins to Regulated Mitochondrial Biogenesis

Sirtuin1 is thought to play a role in regulation of mitochondrial biogenesis. It has been demonstrated in vitro, that polyphenolics, particularly resveratrol, can enhance the activity of the recombinant human sirtuin coded by SIRT1, apparently by a conformational change to the enzyme. Resveratrol at 10 μM also extended the lifespan of yeast from ~23 to ~37 generations (Howitz et al., 2003). Chemical derivatives of resveratrol appear to be even more effective (Yang et al., 2007), suggesting that these compounds in some way decrease the DNA damage associated with aging. These enzyme-activation results have been questioned by subsequent studies on the grounds that resveratrol required highly supra-physiological concentrations (a 3-fold activation at 20 μM) and a non-physiological substrate to have a measurable effect (Grubisha et al., 2005; Kaeberlein et al., 2005). Observations that the plasma concentration of resveratrol from a realistic dose is in the nanomolar range and that it exists in vivo almost entirely as conjugates, rather than as free resveratrol (Goldberg et al., 2003) cast further doubt on sirtuin activation as a key mechanism in vivo. It appears more likely that direct sirtuin activation is only a very minor mechanism

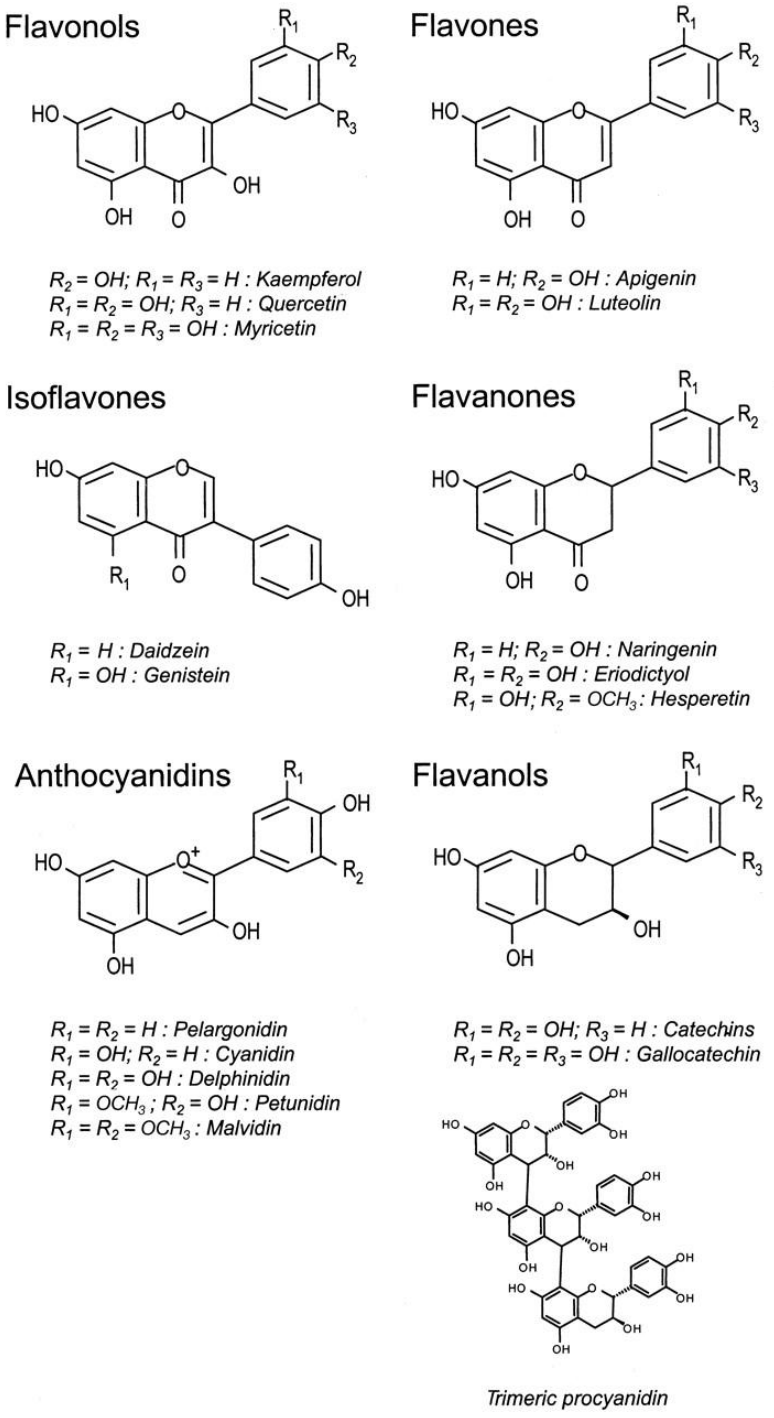


Figure II-5. Chemical structures of flavonoids (Manach et al., 2004).

of mitochondrial biogenesis stimulation by polyphenols in vivo. Furthermore, Park et al. (2012) has showed evidence that resveratrol mediated mitochondrial biogenesis through AMPK-SIRT-PGC-1α pathway with it ability inhibiting PDE and increase cAMP level in skeletal muscle (Figure II-6).

b. Polyphenol Decrease ROS to Regulated Mitochondrial Biogenesis

It is well-established that inhibitors of the ETC increase ROS (Reactive oxygen species) generation. It has been shown, in vitro, that flavonoids can inhibit specific mitochondrial functions, including NADH oxidase (Hodnick et al, 1986), F1-ATPase (Gledhill et al., 2007) and the membrane permeability transition (Santos et al, 1998). Other in vitro studies found that polyphenols inhibited overall mitochondrial respiration (Hodnick et al., 1987) and the closely related rate of ATP generation (Dorta et al., 2005). The former study also detected a burst of ROS generation associated with the inhibition of respiration. A wide range of compounds was tested in these studies, to the extent that structure-activity relationships were established. The two best classes of compound appeared to be stilbenes (e.g., resveratrol) and flavonols (e.g., quercetin). These findings suggest that polyphenols, if they were able to access the mitochondriain vivo, could directly but transiently increase ROS generation, thereby inducing beneficial adaptations in a similar way to exercise.

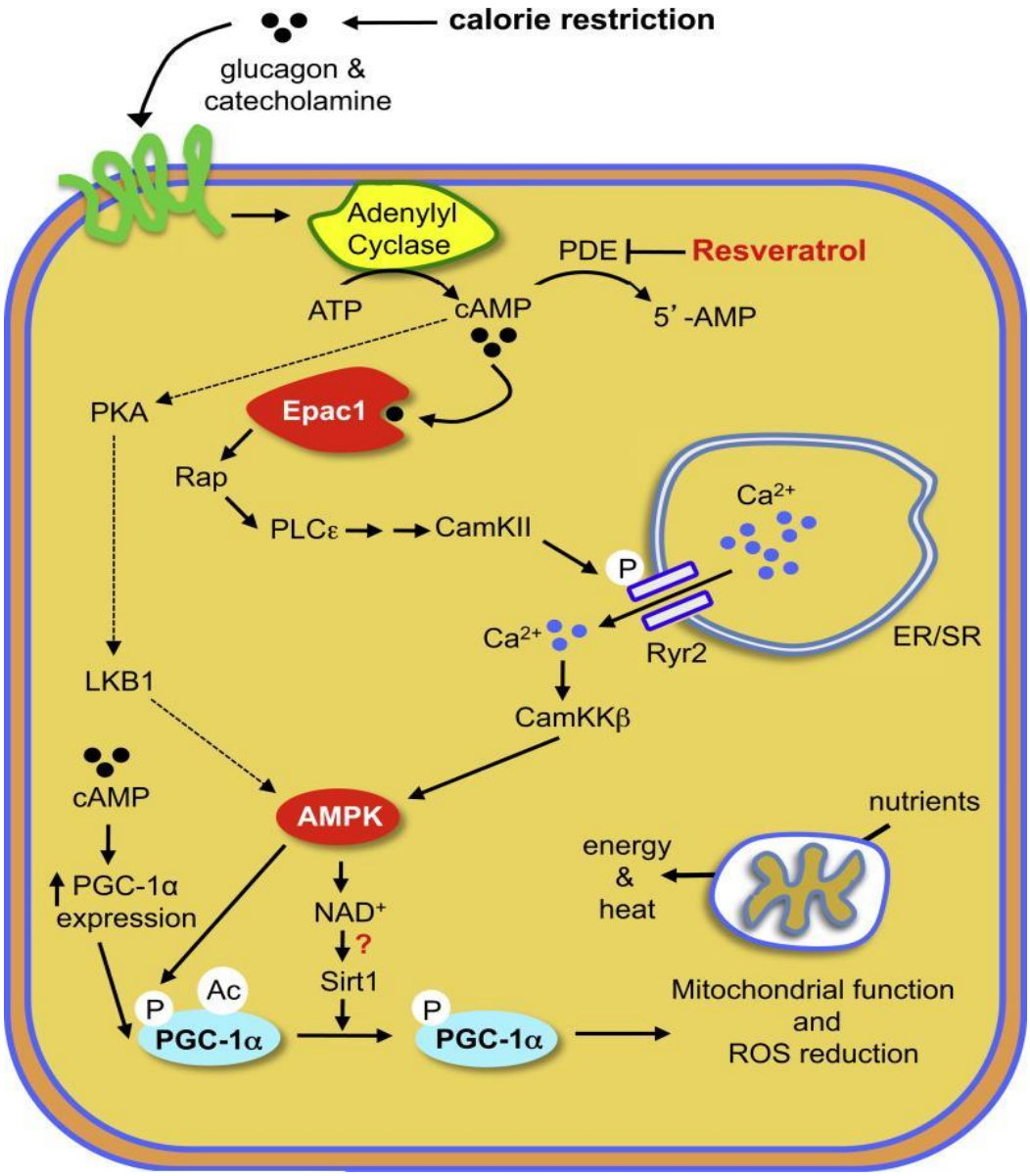


Figure II-6. Proposed model of how polyphenol resveratrol mimics calorie restriction (Park et al., 2012).

c. *Polyphenol Inhibit PDE Activity and Induces cAMP Signaling to Increase Mitochondrial Biogenesis*

Resveratrol inhibits PDE activity and induces cAMP signaling via Epac1, which activates PLCε, resulting in Ca^{2+} release via the Ryr2 Ca^{2+} channel and, ultimately, the activation of the CamKKβ-AMPK pathway. CR increases cAMP levels by increasing glucagon and catecholamine levels, which activate AC activity and cAMP production. AMPK increases mitochondrial biogenesis and function by increasing PGC-1α expression, NAD^{+} levels, and SIRT1 activity. An additional pathway that may contribute to resveratrol action is indicated with dotted lines Resveratrol, a polyphenol in red wine, has been reported as a calorie restriction mimetic with potential antiaging and antidiabetogenic properties. Park et al. (2012) was reported that the metabolic effects of resveratrol result from competitive inhibition of cAMP-degrading phosphodiesterases, leading to elevated cAMP levels. Furthermore, this study was indicated that resveratrol inhibits PDE4 in order to increase cAMP and finally through AMPK-SIRT1-PGC-1α pathway regulated mitochondrial biogenesis (Figure II-6). Similar with that study Dallas et al. (2008) also indicated that polyphenol SINETROL have ability to inhibit PDE in human body fat adipocytes to show strong lipolytic effect mediated by cAMP-PDE inhibitor. This result together seems look like that polyphenol have ability to regulated mitochondrial biogenesis with inhibit PDE activity to increase cAMP levels.

C. Effect of Polyphenol Curcumin in Skeletal Muscle

a. Curcumin

Turmeric is a spice stemming from the rhizomes belonging to a ginger family (Zingiberaceae) component called Curcuma longa. Described as horizontal underground stems with shoots and leaves, rhizomes are notable for their vibrant yellow colour. This colouris largely derived from fat-soluble polyphenolic pigments called curcuminoids). The yellow pigment segregated from from the rhizomes of Curcuma longais termed curcumin, or turmeric, as it is the natural phenol curcuminoid. The structure of curcumin was established in the year 1910 and it was originally isolated almost two hundred years ago. The main constituent of turmeric (a spice) is curcumin, which is similar to aspirin (a renowned carcinogenesis prevention chemical) in its chemical makeup. The dietary phytochemical curcumin (chemical structure shown in Figure II-7) has a long his history of medicinal use in India and Southeast Asia for a wide variety of medical condition. The safe consumption of curcumin is easily confirmed by the fact that for hundreds of years it has frequently been part of the diet of people in a number of countries. Curcumin was reported to have antitumor, antioxidant, antiarthritic and anti-inflammatory properties. Docking studies of these were performed using GOLD and AutoDock into a few well validated targets of anticancer therapy (COX-2, PhenolsulphoTransferases, Matrix metalloproteinases (MMPs), P450 and TNF-alpha) has showed a good correlation was observed in binding affinity of BDC a gainst the targets

.

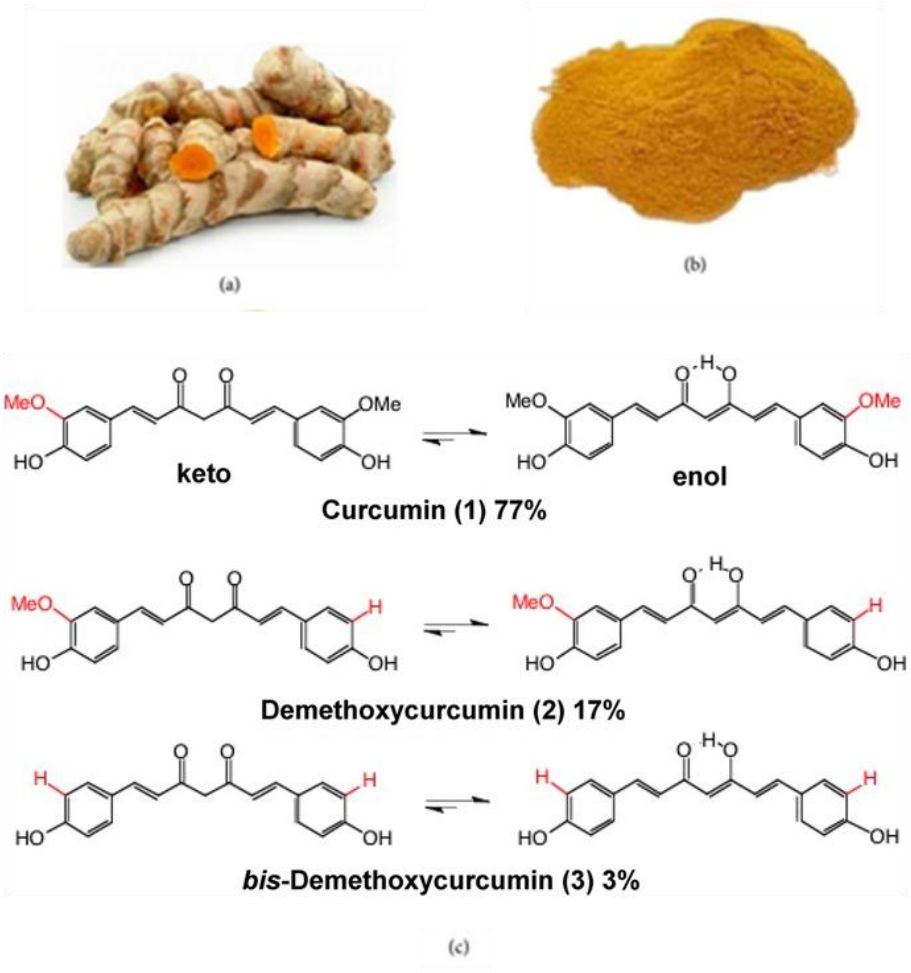


Figure II-7. Turmeric, curcumin and its chemical structure (Shetty et al., 2014) (a) The root of turmeric. (b) Crystallized powder of curcumin. Curcumin is thought to be the main active ingredient derived from the root of turmeric. (c) The enol and keto forms of curcumin are common structures of the drug. The enol form is more energetically stable in the solid phase and in solution.

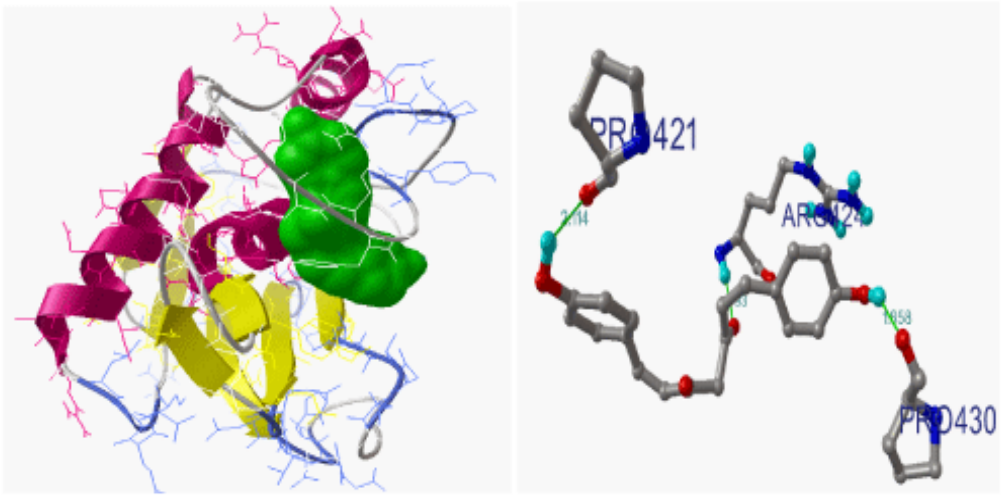


Figure II-8. Binding mode of BDC (bis-Demethoxycurcumin) (Girija et al., 2010)
Binding mode of BDC (bis-Demethoxycurcumin) (shown in Green Molecular Surface model) to MMPs (Left). Binding mode of BDC in the active site (top view) of MMPs along with interacting amino acids(Right) from their respective regions of active site.

indicating these derivatives are potent procarcinogen activating enzyme inhibitors (Figure II-8). Curcumin, a hydrophobic natural product, comprises two phenolic rings. Each ring is replaced with methoxy ether functionality in the ortho-position and attached to each other by an aliphatic unsaturated heptene linker in the para-position with an α, β diketonic functionality on carbon-3 and -5. The electrophilic α, β-unsaturated carbonyl groups are capable of reacting with a nucleophile such as glutathione (Scapagnini et al., 2002). Results from a number of studies suggest that the diketone functionality is able to go through reversible tautomerization between enolic- and ketonic-forms (Payton et al., 2007). Commercial curcumin characteristically comprises three main curcuminoids: curcumin (~77%), demethoxy curcumin (~17%) and bis-demethoxycurcumin (~3%) (Figure II-7). Curcumin is radiant yellow in colour at pH 2.5 to 7 and evolves to red at pH ≥7. Whether in solid or soluble form, curcumin goes through photo degradation upon exposure to light. Curcumin has a melting point of 183^{o}C, a molecular formula of C21H20O6, and a molecular weight of 368.37 g/mol. It is not as soluble in water as in organic solvents such as dimethyl sulfoxide, ethanol, methanol and acetone.

b. Effect of Curcumin to Increase Mitochondrial Biogenesis

Curcumin may surpass other bioactive components with regard to potential, but because of its low bioavailability its usefulness and attainable blood levels associated to its consumption is laced withdoubt (Kidd, 2009). A previous study had shown that the anti-oxidant activities of curcumin were more potent than

those of another polyphenol, resveratrol (Aftab et al., 2010). Furthermore, the long-term effects of dietary curcumin on various markers of mitochondrial biogenesis have also been investigated. Five-month dietary supplementation of curcumin has been shown to up-regulate PGC-1α protein expression in senescence-accelerated mouse-prone 8 (SAMP8), a fast-aging mouse strain, thereby improving mitochondrial membrane potential (MMP) and ATP levels and restoring mitochondrial fusion in the brain (Eckert et al, 2013). However, the effects of curcumin on the skeletal muscle and its underlying mechanisms in the regulation of mitochondrial biogenesis are yet to be elucidated.

III. Research Design and Structure

Muscle adaptation occurs in the mitochondria following exercise training. Endurance exercise training has the potential to enhance metabolic characteristics in the skeletal muscle, including mitochondrial biogenesis. Several polyphenols have been shown to activate cAMP, and are currently under intense investigation as potential inducers of mitochondrial biogenesis. The anti-oxidant activities of curcumin have been reported to be more potent than those of another well-studied polyphenol, resveratrol and the long-term effects of dietary curcumin on various markers of mitochondrial biogenesis have been investigated. The effects of curcumin on the regulation of mitochondrial biogenesis in skeletal muscle however, have yet to be elucidated. The primary purpose of the present study was to determine the combined effects of curcumin treatment together with 24 days of eTR on the regulation of mitochondrial biogenesis in skeletal muscles (Gas and Sol). We predicted that treatment with curcumin combined with endurance training (eTR), may additively or synergistically enhance the effect of exercise. In the order to clarify this hypothesis, present study aimed to examine the effects of combination of endurance training (eTR) and curcumin treatment on the expression of AMPK, SIRT1, PGC-1α, and OXPHOS subunits, mitochondrial DNA copy number, and CS activity in rat skeletal muscle. Furthermore, the present study also examined the effect of exercise and curcumin treatment on the levels of cAMP and downstream targets of PKA including

phosphorylated CREB and LKB-1. The schematic schedule for present experiments shown on diagram at Figure III-1.

1. Effect of Curcumin Treatment on Mitochondrial Marker (Experiment I)

Previous studies have showed evidence that endurance exercise have ability to increase mitochondrial biogenesis in skeletal muscle. Curcumin have been investigated can increase mitochondrial biogenesis in brain and liver. However, effect curcumin treatment itself and together with endurance exercise have not yet elucidated. On the experiment I, we used multiple parameters to determine whether mitochondrial biogenesis occurring in the curcumin treatment in skeletal muscle including mitochondrial marker COX-IV and OXPHOS sub unit expression. Furthermore we also examined mitochondrial DNA copy number and Citrate Synthase (CS) activity.

2. Mechanism Pathway How Curcumin Treatment Regulated Mitochondrial Biogenesis (Experiment II)

PGC-1α has been extensively described as a master regulator of mitochondrial biogenesis. Two metabolic sensors, AMPK and SIRT1 have been described to directly affect PGC-1α activity through phosphorylation and deacetylation, respectively. To determine how mechanism pathway curcumin treatment increases mitochondrial biogenesis, we examined curcumin treatment on AMPK-SIRT1-PGC-1α pathway included phosphorylation AMPK, SIRT1 expression and NAD/NADH ratio, and deacetylation of PGC-1α.

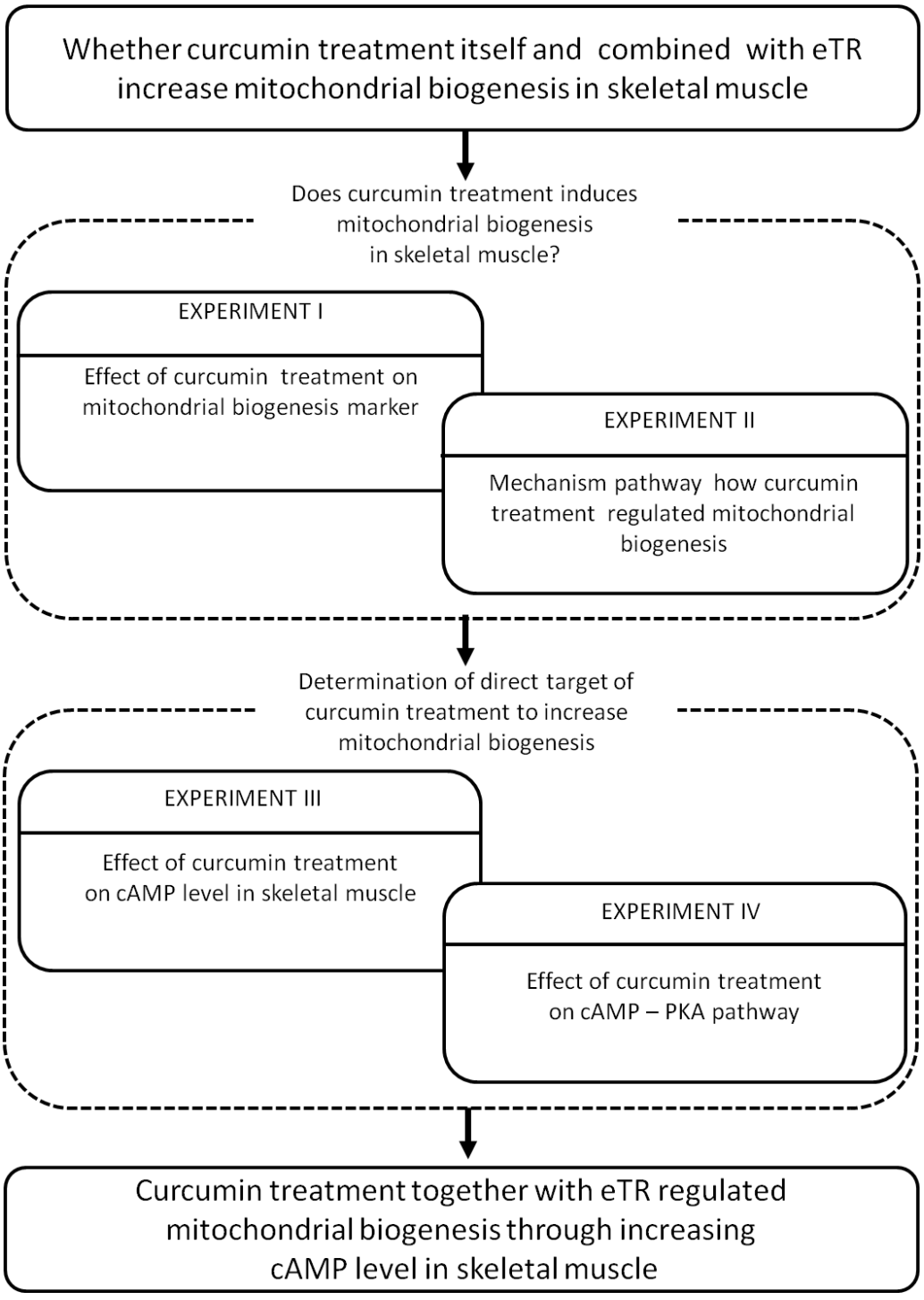


Figure III-1. Content of the present study.

3. Effect of Curcumin Treatment on cAMP Level in Skeletal Muscle (Experiment III)

In both mammalian cells and yeast, the regulation of mitochondrial biogenesis clearly involves the cAMP signaling pathway. In the experiment III, our purpose is to examine the direct target of curcumin treatment to increase mitochondrial biogenesis. To clarify this purpose, we examined curcumin treatment on cAMP level on gastrocnemius.

4. Effect of Curcumin Treatment on cAMP - PKA Pathway (Experiment IV)

Protein kinase A (PKA), also known as cAMP-dependent enzyme, is a well-studied, downstream effector of cAMP and is activated only in the presence of cAMP. To determine whether cAMP needed or involve on curcumin treatment induces mitochondrial biogenesis, we used PKA inhibitor (H89) to determine this effect. Furthermore, we examined downstream target of PKA included LKB-1, CREB, AMPK, PGC-1α and COX-IV.

IV. Effect of Curcumin Treatment on Mitochondrial Marker (Experiment I)

1. Purpose

It is well established that exercise training induces an increase in muscle mitochondria, resulting in fatigue resistance and enhanced endurance performance (Holloszy, 1967). One of the main adaptations of skeletal muscle to endurance training is an increase in oxidative capacity by stimulation of mitochondrial biogenesis. Oxidative phosphorylation is the main source of energy, in the form of ATP, during active steady state in most types of muscle fibres at exercise. During transition from rest to work, the increased ATP demand must be matched by an elevated ATP supply in order to prevent fast complete exhaustion of ATP, which would lead to termination of exercise and possibly to muscle-cell death. In subsequent steps, exercise leads to the induction of proteins that control the transcriptional regulation of the OXPHOS system and the transcription and replication of mitochondrial DNA (Canto et al., 2007). This present experiment we examined whether curcumin treatment itself and curcumin treatment together with endurance exercise increase mitochondrial marker COX-IV, OXPHOS subunit expression, mitochondrial DNA copy number and CS mitochondrial enzyme activity. Our hypothesis said that curcumin itself increase mitochondrial biogenesis marker and augment endurance exercise effect to increase mitochondrial biogenesis marker.

2. Methods

A. Animals

All procedures performed in the present study were approved by the Ethics Committee on Animal Experimentation of Kanazawa University (Protocol: AP-10187). Ten-week-old male Wistar rats (body weight, 282–375 g) were used for the experiments. The animals were housed in an air-conditioned room and exposed to a 12-h light-dark photoperiod. A standard diet (Oriental Yeast, Tokyo, Japan) and water were provided *ad libitum*. The animals were randomly divided into six groups: control without eTR group, curcumin 50 mg/kg-BW/day without eTR, curcumin 100 mg/kg-BW/day without eTR, control with eTR group, curcumin 50 mg/kg-BW/day with eTR, and curcumin 100 mg/kg-BW/day with eTR. Low doses of curcumin (50 mg/kg-BW/day) or high doses of curcumin (100 mg/kg-BW/day) were dissolved in dimethyl sulfoxide (DMSO). All animals were intraperitoneally injected once daily for twenty-eight days with DMSO solution containing curcumin or the same volume of DMSO (vehicle alone).

B. Endurance Exercise Training

The endurance exercise training (eTR) group rats swam 2 h/day in four 30-min bouts separated by 5 min of rest. After the first 30-min bout, a weight equal to 2% of body weight was tied to the body of the rats. The rats swam with the weight attached for the remaining three exercise bouts. All rats swam in a barrel filled to a depth of 50 cm. The swimming area was 190 cm^2/rat (Kawanaka et al.,

1985). The rats performed the above swimming protocol once a day for 24 days (6 days/week × 4 weeks).

C. Sample Preparation

Animals were anesthetized after one hour last endurance exercise session with 50 mg of pentobarbital sodium per 100 g of body weight. For biochemical studies, two calf muscles (m. gastrocnemius [Gas] and the m. soleus [Sol]) were quickly isolated; the tissues were washed in ice-cold saline, separated from the connective tissues and nerves, and then frozen in liquid nitrogen. Nuclear proteins were isolated using a modified version of the protocol established by Blough (Blough et al., 1999). Approximately 40 mg of muscle was homogenized in 500 μl of ice-cold buffer A (250 mM sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 1 mmol/l dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μl/40 mg tissue protease inhibitor cocktail) on ice for ~30 s. The homogenate was then centrifuged (Centrifuge 5415R, Eppendorf, Germany) for 5 min at 500 *g* at 4°C. The supernatant, representing a crude fraction, was used as the total tissue fraction in the immunoblots. The remaining pellet was resuspended in 500 μl of ice-cold buffer B (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 50 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and 2 μl/40 mg tissue protease inhibitor cocktail) and placed on ice for 10 min, with occasional mixing. The resuspended pellet was spun in a centrifuge for 5 min at 3000 *g* at 4°C. The supernatant, representing the nuclear fraction, was extracted and stored.

D. Western Blotting

Western blotting analysis was performed as previously described (Furuichi et al, 2010). Briefly, equal protein amounts of samples were loaded onto SDS-PAGE (SDS-polyacrylamide gel electrophoresis) gels 12.5% (COX-IV and Total OXPHOS) and the proteins were transferred onto a polyvinylidene difluoride membrane (PVDF; polyvinylidene difluoride membrane, ATTO, Japan). The membrane was incubated in Tris-buffered saline (TBS) buffer (150 mm NaCl, 25 mm Tris-HCl, pH 7.4) containing 0.1% Tween-20 for 10 min at room temperature. The membrane was then incubated on 4% blocking buffer on room temperature for 1 hour. The membrane was then incubated with mouse monoclonal antibody to COX-IV (1:1000 dilution, #: ab14744-GR125134-4, Abcam, Cambridge, England), Total OXPHOS Rodent WB antibody Cocktail (1:1500 dilution, #: MS604-G2830, Abcam, Cambridge, England), Mouse monoclonal antibody to β-actin (1:1000 dilution, #: ab8226-GR110198-3, Abcam, Cambridge, England), Mouse monoclonal to GAPDH (1:1000 dilution, #: ab8245-GR168547-1, Abcam, Cambridge, England) antibodies at 4°C for overnight and rinsed with TBS-T 3 times. It was then reacted with HRP (HRP; horseradish peroxidase-conjugated) Anti-rabbit IgG, HRP-linked Whole Ab donkey (Peroxidase-Linked Secondary Antibodies, GE healthcare, England) or Anti-mouse IgG HRP-linked Whole Ab donkey (Peroxidase-Linked Secondary Antibodies, GE healthcare, England) and signals were visualized by the enhanced chemiluminescence detection method using the ECL Plus Western

blotting detection system (GE Healthcare, USA). The signal intensity was quantified using imaging software (Image J, version 1.46, NIH, Maryland, USA).

E. Mitochondrial DNA Copy Number

Genomic DNA was extracted using a spin column. Briefly, muscle tissue was treated with proteinase K (20 µl)(Qiagen, Netherland), followed by addition of SDS (200 µl) to lyse the cells. The homogenized solution was incubated at 56°C and 100% ethanol (200 µl) was added to precipitate the DNA. The mixture was applied to a QIAamp spin column, and after two washes with buffer (500 µl), genomic DNA was eluted with elution buffer (200 µl). The final DNA concentration was determined by UV spectrophotometry at 260 and 280 nm. The mitochondrial (mt) copy number was estimated by quantitative real-time PCR (ABI 7300 Real Time PCR System; Foster City, CA) from the relative amounts of nuclear and mtDNA. (mt:nuclear DNA) which reflects the concentration of mitochondria per cell in the tissue. The mt.DNA forward primer used was (5' to 3') CCCTAAAACCCGCC ACATCT and the reverse primer was GAGCGATGGTGAGAGC TAAGGT. For nuclear DNA, the forward primer was CGAGTCGTCTTT CTCCTGATGAT and the reverse primer was TTCTGGATTCCAA TGCTTCGA. Quantification was performed in a total reaction volume of 20 µl containing: 2X SYBR Tli RNaseH Plus (10 µl) (Takara Bio, Japan) each primer (0.8 µl), 50X ROX Reference Dye II (0.4 µl) (Takara Bio, Japan) sample DNA (2 µl), and water (6 µl). Amplification and detection were performed in a 7300 real time PCR (Applied Biosystem) system. PCR was

initiated with 95°C denaturation followed by an enzyme activation step for 30 s followed by 40 cycles of 95°C denaturation for 5 s, and annealing and elongation for 30 s at 60°C. Samples were assayed in duplicate. Data analysis was based on measurement of the cycle threshold (CT), and the difference in CT values was used as a measure of relative abundance: CT(nDNA) −CT(mt.DNA) or ΔCT, a quantitative measure of the mitochondrial genome. Results were expressed as the copy number of mtDNA per cell, $2 \times 2^{-\Delta CT}$, which is a unitless ratio.

F. Citrate Synthase Activity

Skeletal muscle (20 mg) was homogenized on ice in 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.0. Citrate synthase (CS) activity was determined spectrophotometrically according to the method described by Srere (Srere, 1969). The homogenates were frozen and thawed thrice to disrupt the mitochondria to expose the CS and the assay was performed in a total volume of 900 µl: 100 mM Tris buffer (pH 8.0), 1 mM 5,5-dithiobis (2-nitrobenzoate) (DTNB), 3 mM acetyl-CoA, 5 mM oxaloacetate (OAA), and 100 µl of muscle homogenate. The principle of the assay is to initiate the reaction of acetyl-CoA with OAA and link the release of free CoA-SH to a colorimetric reagent, DTNB. All measurements were performed in duplicate at 30°C and read used spectrophotometri (Lambda25, Perkin Elmer, USA) on 412 nm. CS activity was normalized to the total protein content and was expressed as micromoles per gram protein per minute.

G. *Statistical Analysis*

Two-way analysis of variance (ANOVA) as used to assess the main effect of exercise (non-eTR vs. eTR groups) and curcumin (non-curcumin vs. curcumin groups) and the exercise x curcumin interaction. The Tukey-Kramer post-hoc test was used for analysis to identify of difference between low dose and high dose curcumin groups. All data were expressed as mean ± standard deviation (SD). The level of significance was established at $P < 0.05$.

3. Result

A. Effect of Curcumin Treatment on Body Weight Gain.

Descriptive data for body weight are presented in Table IV-1. Two-way ANOVA indicated that eTR decrease weight gain compare non-eTR ($F = 45.91$, $P < 0.001$). Furthermore rats with curcumin treatment also decreased the weight gain compare non-curcumin group ($F = 15.85$, $P < 0.001$) and no interaction between two factor ($F = 3.90$, $P > 0.05$). Post-hoc test indicated that only high dose curcumin treatment significant different than DMSO ($P < 0.05$) These results indicated that curcumin additive eTR effect to reduce body weight gain.

B. Curcumin Treatment with eTR Enhances Expression of The Mitochondrial Markers COX-IV and OXPHOS Subunit

Owing to the complementary effects of curcumin and eTR on AMPK phosphorylation, SIRT1 expression, NAD^+/NADH ratio, and deacetylation of PGC-1α, we hypothesized a potentially additive or synergistic effect of the two

parameters on mitochondrial biogenesis. We assessed the mitochondrial content in skeletal muscle by measuring the expression level of cytochrome c oxidase subunit IV (COX-IV). Figure IV-1 and IV-2 show the change in the COX IV protein expression in skeletal muscle after 28 days of treatment with eTR or curcumin treatment. Two-way ANOVA showed significant effect for eTR in Gas and Sol ($F = 173.03$ for Gas, 150.75 for Sol, $P < 0.001$) and curcumin treatment ($F = 36.94$ for Gas, 20.77 for Sol, $P < 0.001$) and no interaction between two factor (n.s.). Furthermore, post-hoc test analysis indicated that both doses of curcumin significant different than DMSO ($P < 0.05$) and high dose and low dose were significant different ($P < 0.05$). The results of four representative oxidative phosphorylation (OXPHOS) subunits by two-way ANOVA analysis demonstrated that eTR upregulated the protein levels, in both Gas and Sol (Figure IV-3A & C, IV-4A & C, $P < 0.001$) but was not showed for Complex V: ATP synthase subunit-α (Figure IV-3B and IV-4B, n.s.). Furthermore, in both Gas and Sol, curcumin treatment increase Complex I subunit NDUFB8 ($F = 16.92$ for Gas, 131.71 for Sol, $P < 0.001$) and no interaction between two factor (n.s.), Complex II subunit 30 kDa ($F = 10.59$ for Gas, 13.68 for Sol, $P < 0.001$) and no interaction between two factor (n.s.), and Complex III subunit Core 2 ($F = 35.33$ for Gas, 60.229 for Sol, $P < 0.001$) and no interaction between two factor (n.s.), whereas levels of Complex V: ATP synthase subunit-α was not increased in Gas and Sol (n.s.). Addition, post-hoc analysis indicated that high dose curcumin significant different than low dose ($P < 0.05$) to increase OXPHOS subunit Complex I, II, III and COX-IV in Gas and Sol ($P < 0.05$).

	Body Weight		
GROUPS	Before Exercise (g)	After Exercise 28 days (g)	△
DMSO	352.3 ± 9.6	399.2 ± 13.6	46.8 ± 5.8
CD 50	341.5 ± 32.3	380.8 ± 31.2	39.3 ± 32.8
CD 100	387.3 ± 14.2	420.2 ± 13.6	32.8 ± 5.2
DMSO + eTR	330.1 ± 35.6	359.3 ± 35.7	29.3 ± 4.5
CD 50 + eTR	331.2 ± 26,6	364.7 ± 26.1	33.5 ± 5.5
CD 100 + eTR	335.8 ± 18.7	359.3 ± 13.2	21.5 ± 6.1

Table IV-1. Descriptive data for body weight
Value are mean ± SD (n = 6 in each group) DMSO = control without endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. DMSO + eTR = control with endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO + endurance exercise. CD 100 = curcumin 100 mg/kg-BW/day in DMSO + endurance exercise. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) no interaction between two factor for body weight gain after 28 day eTR and curcumin treatment. Post-hoc analysis revealed that compared to DMSO group, only high doses curcumin were showed significant decrease body weight gain ($P < 0.05$).

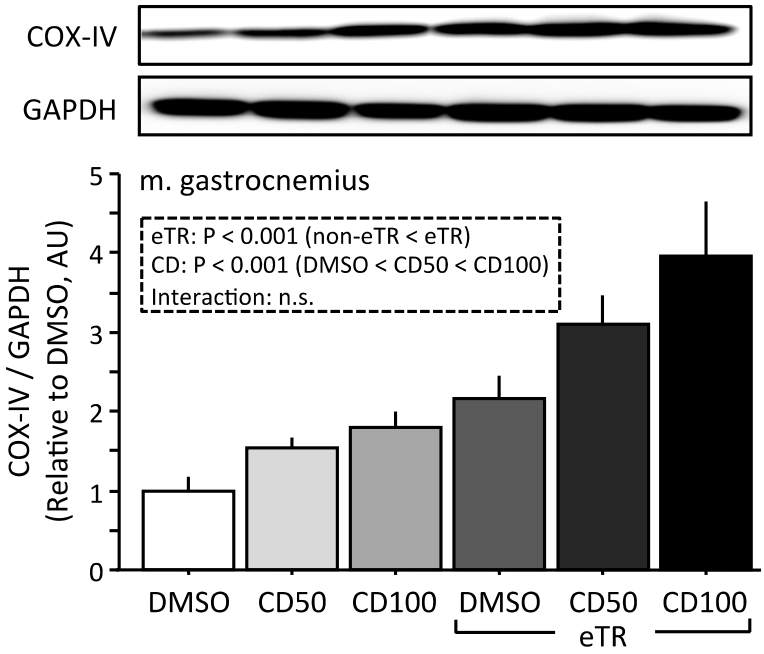


Figure IV-1. Effect of curcumin treatment increases COX-IV protein expression in gastrocnemius.

Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for COX-IV in Gas and no interaction between two factor ($P > 0.05$). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increase COX IV ($P < 0.05$) in Gas.

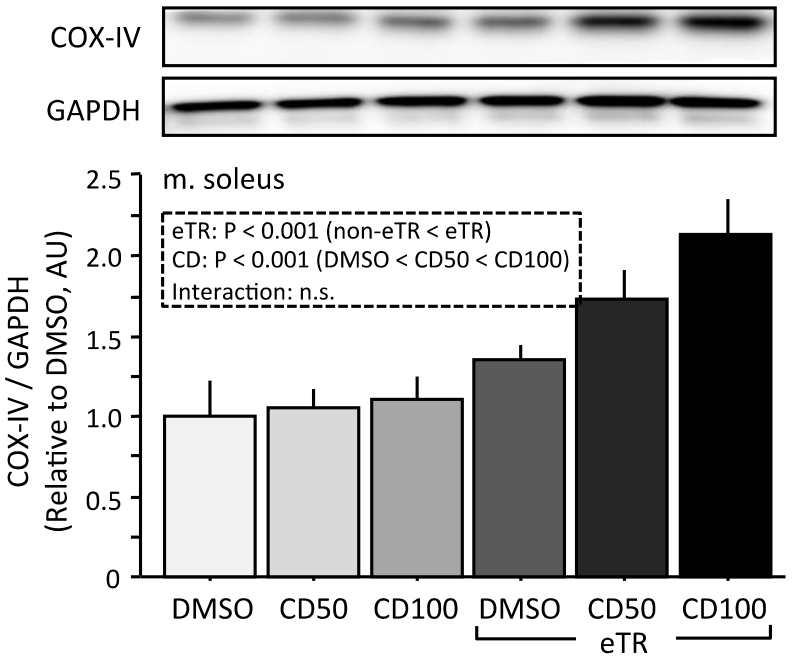


Figure IV-2. Effect of curcumin treatment increases COX-IV protein expression in Soleus.

Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for COX-IV in Sol and no interaction between two factor ($P > 0.05$). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increase COX IV ($P < 0.05$) in Sol.

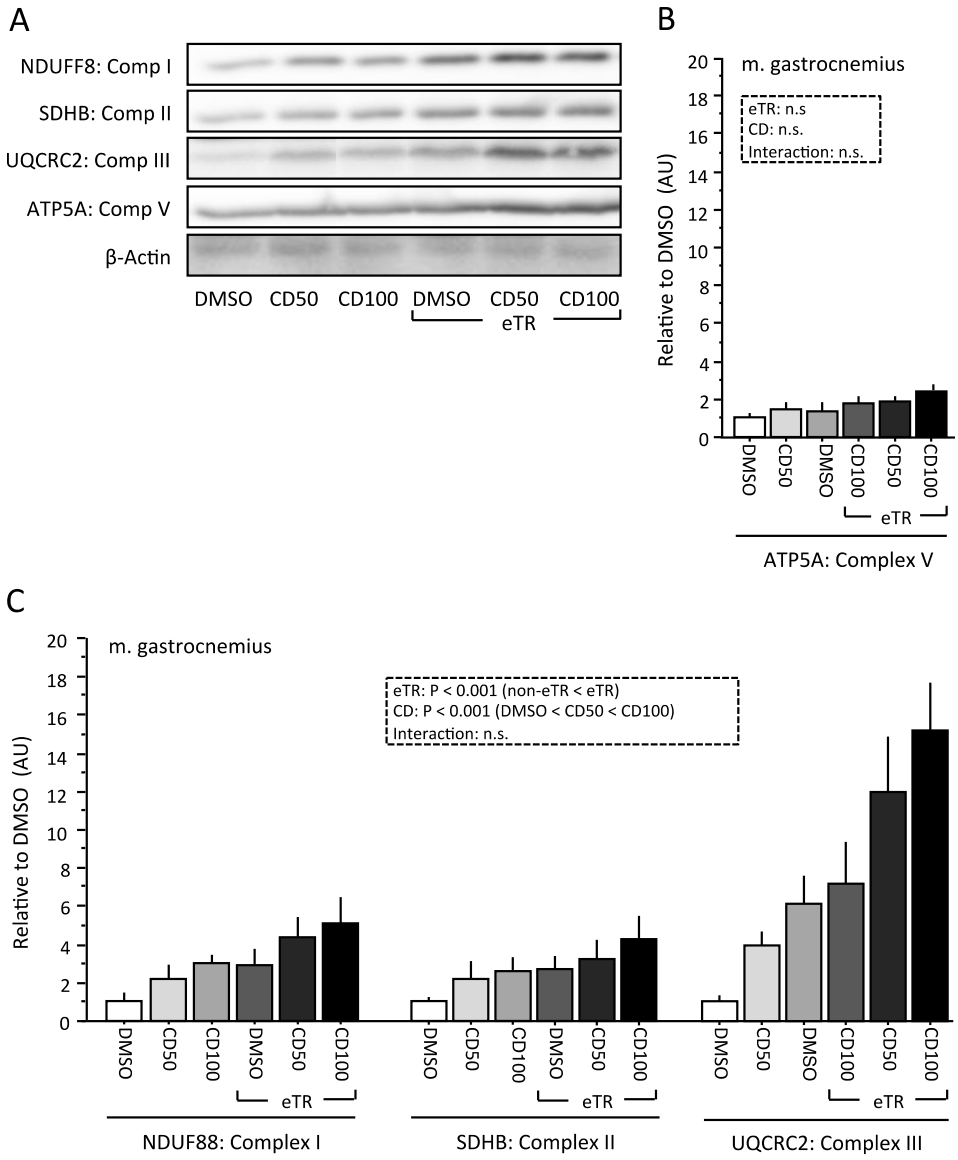


Figure IV-3. Effect of curcumin treatment increases total OXPHOS subunits expression in Gastrocnemius.

Value are mean ± SD (n = 6 in each group) DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for OXPHOS subunit Complex I, II and III (A, C) and no interaction between two factor ($P > 0.05$) but not in Complex V (B) in Gas. Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increases total OXPHOS subunits I, II and III ($P < 0.05$) in Gas.

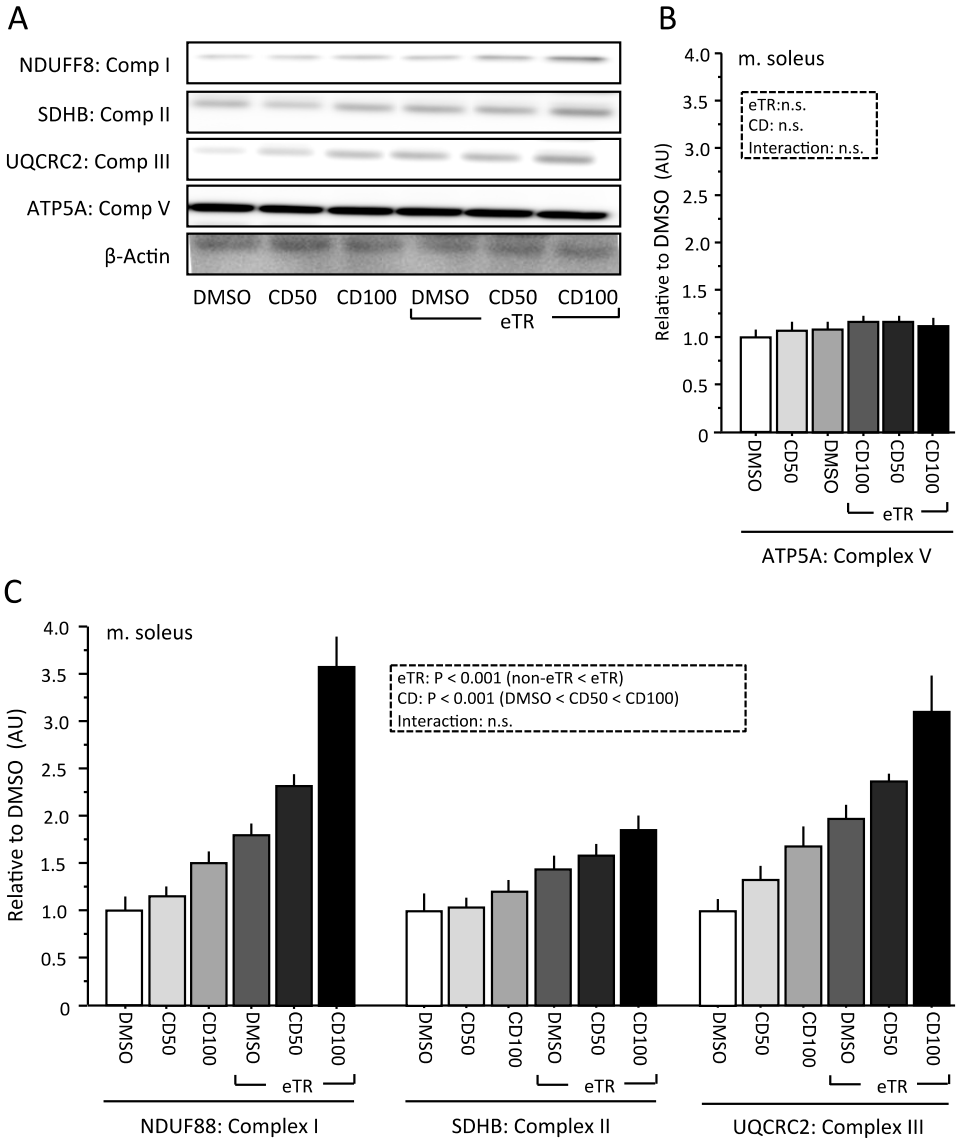


Figure IV-4. Effect of curcumin treatment increases total OXPHOS subunits expression in Soleus.

Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for OXPHOS subunit Complex I, II and III (A, C) and no interaction between two factor ($P > 0.05$) but not in Complex V (B) in Sol. Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increase total OXPHOS subunits I, II and III ($P < 0.05$) in Sol.

In conclusion, these results indicated that high-dose curcumin treatment in conjunction with eTR increased the expression of COX-IV and OXPHOS subunits in skeletal muscle and curcumin and eTR were found to act additively.

C. Curcumin Treatment Together with eTR enhances the Mitochondrial DNA Copy Number and CS Activity

The relative amount of mtDNA in skeletal muscle was determined by RT-PCR. An increase in mtDNA copy number relative to nuclear DNA is one of the best molecular markers of mitochondrial biogenesis (Medeiros, 2008). Two way ANOVA demonstrated that eTR significantly increased the mtDNA copy number in both Gas and Sol compared to non eTR (Figure. IV-5, $F = 593.82$ for Gas, 145.94 for Sol, $P < 0.001$), curcumin treatment increased mtDNA copy number compared to that by the control only DMSO (Figure IV-5, $F = 43.46$ for Gas, 14.05 for Sol, $P < 0.001$) and no interaction between two factor (n.s). Furthermore, post-hoc test analysis indicated that both doses curcumin significant different than DMSO group ($P < 0.05$) in Gas but in Sol only high dose significant different than DMSO ($P < 0.05$). In addition,, eTR increased CS enzyme activity in Gas and Sol (Figure IV-6, $F = 652.69$ for Gas, 152.09 for Sol, $P < 0.001$) and this was observed also with curcumin treatment where the CS activity increased in both Gas and Sol (Figure IV-6, $F = 42.34$ for Gas, 38.04 for Sol, $P < 0.001$) no interaction between two factor (n.s.). Meanwhile, post-hoc analysis result indicated that only high dose curcumin significant different than DMSO ($P < 0.05$) to enhance CS activity in Gas and Sol ($P < 0.05$). These result

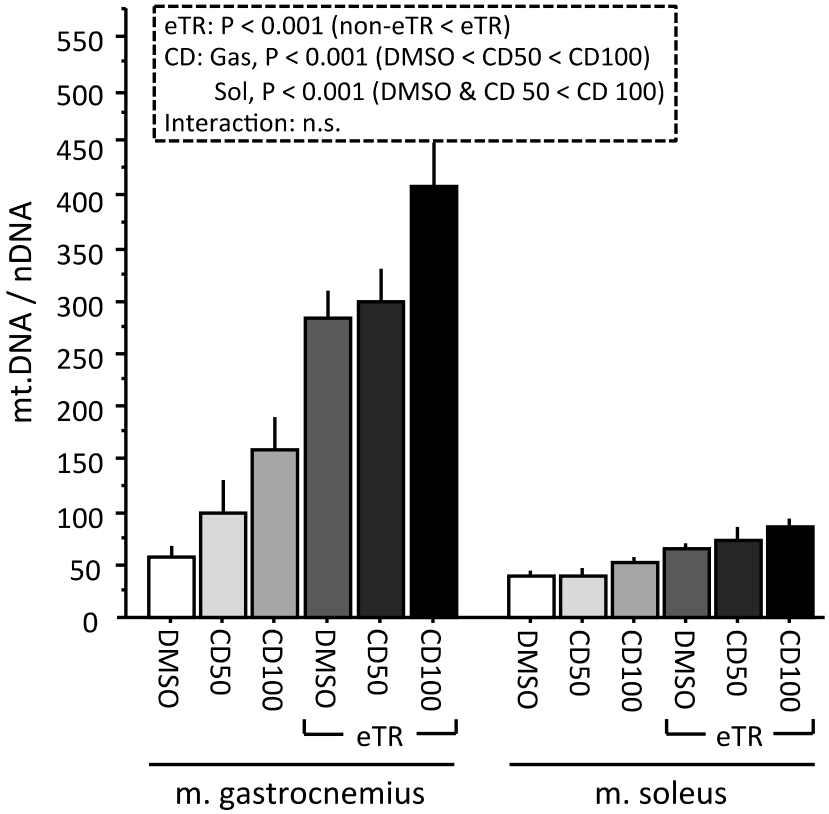


Figure IV-5. Effect of curcumin treatment increases mitochondrial DNA copy number in skeletal muscle.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for mitochondrial DNA copy number in Gas and Sol no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increases mitochondrial copy number in Gas ($P < 0.05$) but in Sol only for high dose curcumin.

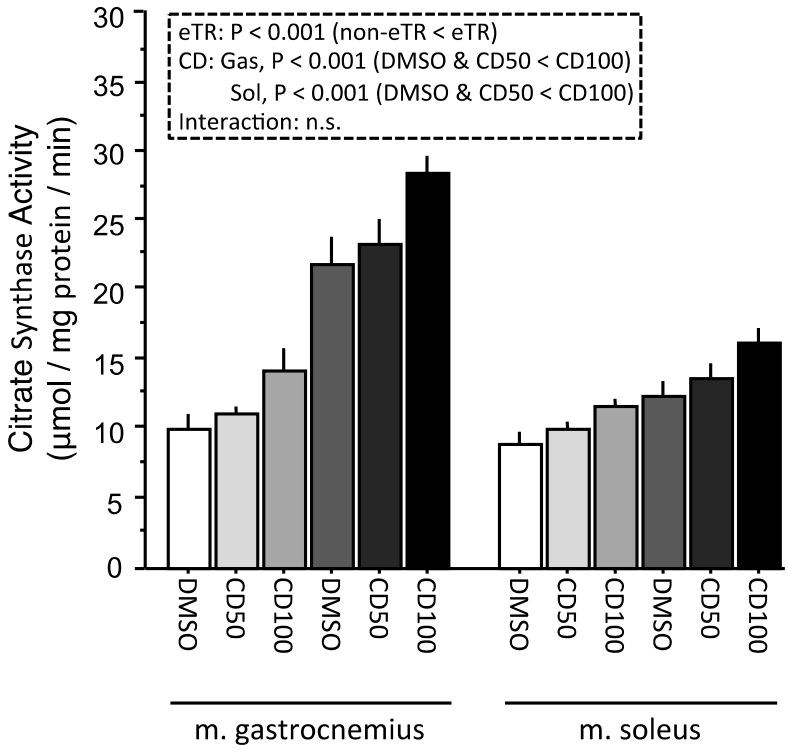


Figure IV-6. Effect of curcumin treatment increases citrate synthase (CS) activity in skeletal muscle.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for CS activity in Gas and Sol, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, high dose curcumin increases CS activity in Gas and Sol ($P < 0.05$)

indicate that high-dose curcumin treatment and eTR additively increases the mitochondrial DNA copy number and CS activity in skeletal muscle.

4. Discussion

Mitochondrial biogenesis is influenced by environmental stress such as exercise, caloric restriction, low temperature, oxidative stress, cell division and renewal and differentiation. Mitochondrial biogenesis is accompanied not only by variations in number, but also in size and mass. The process of mitochondrial biogenesis is complex and requires gene products from both the mitochondrial and the nuclear genomes. For example, complex IV in the respiratory chain, named cytochrome c oxidase complex IV subunit IV (COX-IV). It is well established that physical activity increases mitochondrial content (Baar et al, 2002). These early studies in animals suggested 6-week-old rats subjected to exercise 5 days per week for 3 months showed an increase in skeletal muscle cytochrome *c* concentration, as well as increased activities of key mitochondrial enzymes and OXPHOS (oxidative phosphorylation) (Holloszy, 1967). Similar with this study, our result showed that 28 day swimming exercise increase mitochondrial marker COX-IV and total OXPHOS subunits. Previous research suggested that the polyphenol resveratrol combined with exercise could regulate mitochondria biogenesis through its antioxidant activity (Menzies et al., 2013). Based on that evidence, we suggested that curcumin treatment have ability to increase mitochondrial marker included COX-IV and total OXPHOS subunits in skeletal muscle. Indeed, our result showed that curcumin treatment increase

COX-IV and total OXPHOS subunits and curcumin additive endurance exercise induces COX-IV and total OXPHOS subunits in gastrocnemius and soleus. Regulation of mtDNA copy number is essential for maintaining cellular energy requirements. High-energy requiring cells, such as muscle, require large quantities of ATP and maintain high numbers of mtDNA copy. MtDNA replication and transcription are tightly coupled such that the expression of the mtDNA genes, and hence the generation of ATP through OXPHOS, requires continuous replication of mtDNA (Dickinson et al., 2013). Maximal citrate synthase activity (CS) is routinely used as a marker of aerobic capacity and mitochondrial density in skeletal muscle. Exercise training has resulted in increases in CS ranging from 0 to 100% (Holloszy et al., 1970). Similar with that evidence our result showed that endurance training and curcumin treatment increase mitochondrial copy number and CS activity in skeletal muscle.

5. Summary

These results indicate that high-dose curcumin treatment and eTR additively increases the expression of mitochondria marker COX-IV and total OXPHOS subunit, the mitochondrial DNA copy number and CS activity in gastrocnemius and soleus.

V. Mechanism Pathway How Curcumin Treatment Regulated Mitochondrial Biogenesis

1. Purpose

PGC-1α activity is influenced by various post-translational modifications. AMP-activated protein kinase phosphorylate PGC-1α, modifying its stability and activity (Li et al., 2011). Besides these phosphorylation sites, PGC-1α contains multiple distinct acetylation sites. PGC-1α deacetylation has been demonstrated to occur via SIRT1 in vitro as well as in vivo during exercise induced increase in SIRT1 activity has been described in skeletal muscle (Gurd, 2011) and can be mimicked by polyphenol resveratrol (Lagouge et al., 2006) . Recently, a reduced PGC-1α acetylation was described after a single session of exercise (Canto et al., 2009). In addition, the mechanisms underlying the interaction between PGC-1α deacetylation by SIRT1 and PGC-1α phosphorylation by AMPK have been investigated in detail (Canto et al., 2009).

The purpose of present experiment to determine how the mechanism molecule pathway which involve in mitochondrial biogenesis. We examine the effect of curcumin treatment itself and curcumin treatment together with endurance exercise in both doses to phosphorylation AMPK, NAD+/NAH ratio and deacetylation of PGC-1α on skeletal muscle. We suggested that curcumin treatment increase and additively endurance exercise induces mitochondrial biogenesis through AMPK-SIRT1-PGC-1α pathway.

2. Methods

A. Sample Preparation for Western Blotting

Animals were anesthetized after one hour last endurance exercise session with 50 mg of pentobarbital sodium per 100 g of body weight. For biochemical studies, two calf muscles (m. gastrocnemius [Gas] and the m. soleus [Sol]) were quickly isolated; the tissues were washed in ice-cold saline, separated from the connective tissues and nerves, and then frozen in liquid nitrogen. Nuclear proteins were isolated using a modified version of the protocol established by Blough (Blough et al., 1999). Approximately 40 mg of muscle was homogenized in 500 µl of ice-cold buffer A (250 mM sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 1 mmol/l dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 µl/40 mg tissue protease inhibitor cocktail) on ice for ~30 s. The homogenate was then centrifuged (Centrifuge 5415R, Eppendorf, Germany) for 5 min at 500 *g* at 4°C. The supernatant, representing a crude fraction, was used as the total (whole) tissue fraction in the immunoblots whereas the pellet used for nuclear tissue fraction. Supernatant centrifuged for 30 min at 10.000 g at 4^0C following with second centrifuge for 30 min at 1.000 g at 4^0C and used supernatant as cytosol fraction. The remaining pellet for nuclear fraction was resuspended in 500 µl of ice-cold buffer B (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 50 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and 2 µl/40 mg tissue protease inhibitor cocktail) and placed on ice for 10 min, with occasional mixing. The resuspended

pellet was spun in a centrifuge for 5 min at 3000 *g* at 4°C. The supernatant, representing the nuclear fraction, was extracted and stored

B. Western Blotting

Western blotting analysis was performed as previously described (Furuichi et al, 2010). Briefly, equal protein amounts of samples were loaded onto SDS-PAGE (SDS-polyacrylamide gel electrophoresis) gels 10% (p-AMPKα, t-AMPKα), 7.5% (SIRT1, PGC-1α, Acetylated-lysine) and the proteins were transferred onto a polyvinylidene difluoride membrane (PVDF; polyvinylidene difluoride membrane, ATTO, Japan). The membrane was incubated in Tris-buffered saline (TBS) buffer (150 mm NaCl, 25 mm Tris-HCl, pH 7.4) containing 0.1% Tween-20 for 10 min at room temperature. The membrane was then incubated on 4% blocking buffer on room temperature for 1 hour. The membrane was then incubated with Rabbit polyclonal antibody to Phospho-AMPKα (Thr 172) (1:1000 dilution, #: 2535S-16, Cell Signaling Technology, Danvers, MA, USA), Rabbit polyclonal to AMPKα (1:1000 dilution, #: 26035-6, Cell Signaling Technology, Danvers, MA, USA), Rabbit polyclonal to SIRT1 (1:1000 dilution, #: 13181-1-AP-00014457, Proteintech, Chicago, IL, USA), Mouse monoclonal to PGC-1α (1:500 dilution, #: KP9803-D00151286, Calbiochem, San Diego, CA, USA), Rabbit polyclonal to acetylated–lysine (1:1000 dilution, #: 94415-11, Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (1:1000 dilution, #: ab8226-GR110198-3, Abcam, Cambridge, England) and Rabbit polyclonal antibody to lamin A/C (1:1000 dilution, #: SC-

20681-D0142, Santa Cruz Biotechnology, CA, USA) antibodies at 4°C for overnight and rinsed with TBS-T three times. It was then reacted with HRP (HRP; horseradish peroxidase-conjugated) Anti-rabbit IgG, HRP-linked Whole Ab donkey (Peroxidase-Linked Secondary Antibodies, GE healthcare, England) or Anti-mouse IgG HRP-linked Whole Ab donkey (Peroxidase-Linked Secondary Antibodies, GE healthcare, England) and signals were visualized by the enhanced chemiluminescence detection method using the ECL Plus Western blotting detection system (GE Healthcare, USA). The signal intensity was quantified using imaging software (Image J, version 1.46, USA).

C. Immunoprecipitation

The nuclear fraction was obtained using the same procedure as western blotting. In this preparation, buffer D (20 mM Tris-HCl [pH 7.4], 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM PMSF) was used instead of buffers A and C. The nuclear fraction of protein was divided into 1-ml PBS aliquots. Dynabeads (Invitrogen, CA, USA) were added to the samples, and the samples were subsequently incubated at 4°C for 1 h. The Dynabeads were collected using magnets, and normal mouse immunoglobulin G (nIgG; Santa Cruz, USA) was added to the supernatants and incubated at 4°C overnight. Samples were centrifuged at 7000 *g* at 4°C for 30 s, and aliquots of this supernatant were reacted with primary antibodies against PGC-1α and mouse nIgG at 4°C overnight. Dynabeads were then added and incubated at 4°C

for 2 h. The pellet was collected using magnets and washed with PBS. The final pellet from the immunoprecipitation (IP) was analyzed using SDS-PAGE and immunoblotting with western blotting method.

D. NAD^+/NADH Ratio

The intracellular NAD^+/NADH ratio was assessed using an NAD^+/NADH assay kit (K337-100, Biovision, USA) according to the manufacturer's instructions. Briefly, tissues were lysed with 400 µl of NADH/NAD extraction buffer by freezing/thawing. Extracts were vortexed for 10 s and centrifuged at 16,000 g for 5 min. Supernatants containing NAD^+ and NADH were transferred into a new microcentrifuge tube. To determine the concentration of NADH, the samples were incubated at 60°C for 30 min to disrupt NAD^+. Total NAD^+ (tNAD) and NADH samples were mixed with an NAD cycling enzyme, which converts NAD^+ to NADH at room temperature for 5 min, and incubated with an NADH developing agent at RT for 1 h. The amount of NADH was determined by the absorbance at 450 nm at spectrophotometry (Infinite 200 PRO, Tecan, USA). NAD^+ concentration were calculated by subtracting the NADH concentration from the tNAD value.

E. Statistical Analysis

Two-way analysis of variance (ANOVA) as used to assess the main effect of exercise (non-eTR vs. eTR groups) and curcumin (non-curcumin vs. curcumin groups) and the exercise x curcumin interaction. The Tukey-Kramer post-hoc

test was used for analysis to identify of difference between low dose and high dose curcumin groups. All data were expressed as mean ± standard deviation (SD). The level of significance was established at $P < 0.05$.

3. Result

A. Curcumin Treatment with eTR Increases Phosphorylation of AMPK

In accordance with previous study reports, two way ANOVA demonstrated that administration of curcumin enhanced the phosphorylation of the Thr^{172} residue of the AMPKα catalytic subunit in Gas and Sol (Figure V-1 and V-2, $F = 100.51$ for Gas, 17.99 for Sol, $P < 0.001$). However, in both Gas and Sol, eTR treatment enhanced the increase in AMPK phosphorylation (Figure V-1 and V-2, $F = 383.29$ for Gas, 125.96 for Sol, $P < 0.001$) and no interaction between two factor (n.s.). Meanwhile, post-hoc analysis showed that both doses curcumin significant different with DMSO (Figure V-1 and V-2, $P < 0.05$) and low dose was different than high dose ($P < 0.05$) to increase phosphorylation AMPK in Gas and Sol (Figure V-1 and V-2, $P < 0.05$). This result suggests that curcumin activates AMPK phosphorylation in skeletal muscles and that the effect is in addition to the response to eTR.

B. Curcumin Treatment with eTR Increases NAD^+/NADH Ratio and SIRT1 Expression

In accordance with this approach, two way ANOVA demonstrate that eTR increased the cellular NAD^+/NADH ratio in Gas and Sol (Figure V-3 and V-4, F

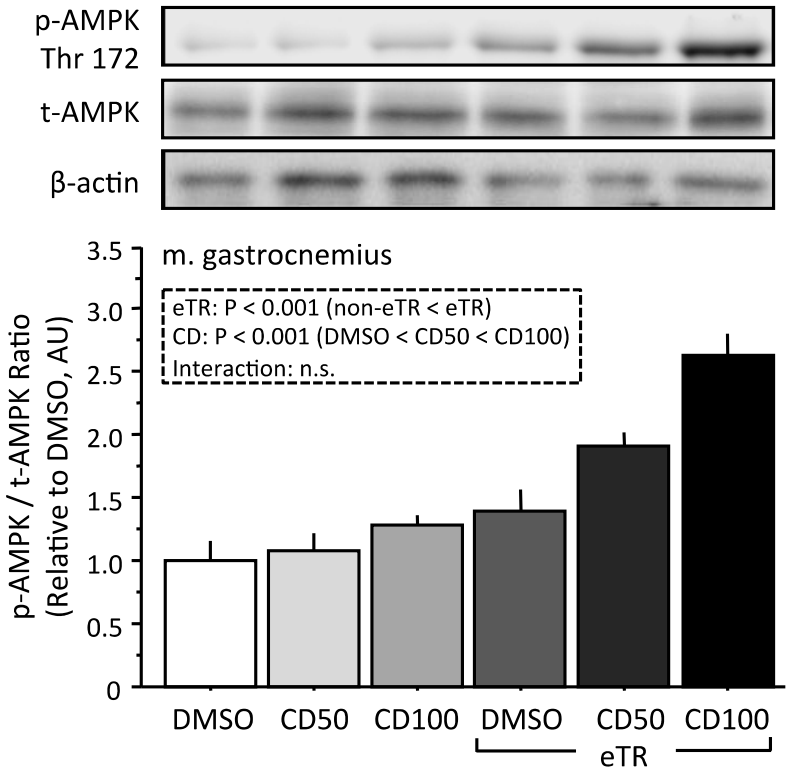


Figure V-1. Effect of curcumin treatment increases phosphorylation of AMPK (Thr 172) in gastrocnemius.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for phosphorylation of AMPK in Gas, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increase phosphorylation of AMPK in gastrocnemius ($P < 0.05$).

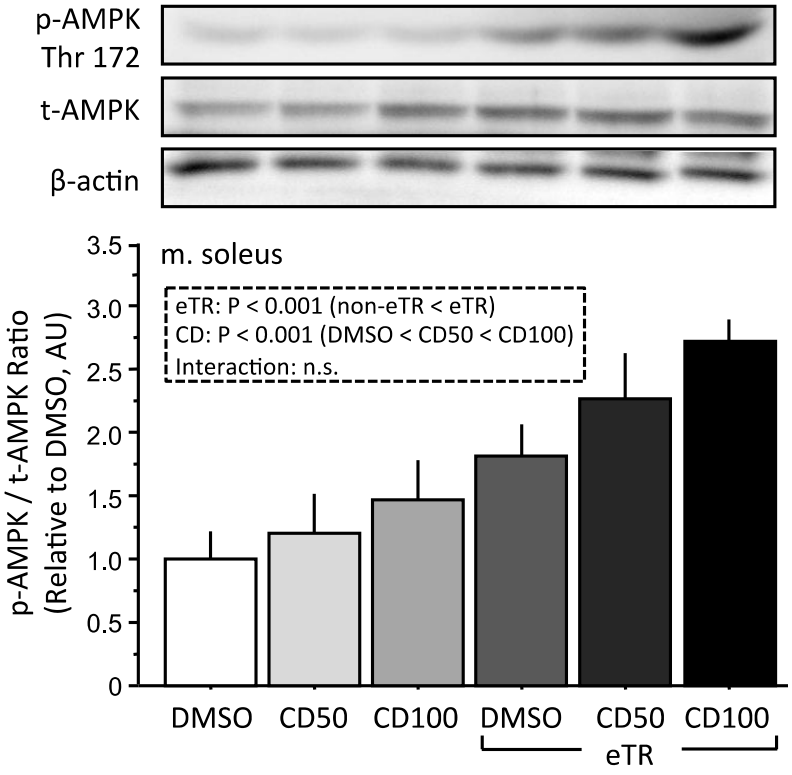


Figure V-2. Effect of curcumin treatment increases phosphorylation of AMPK (Thr 172) in soleus.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for phosphorylation of AMPK in Sol, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increase phosphorylation of AMPK in soleus ($P < 0.05$).

= 243.73 for Gas, 587.07 for Sol, $P < 0.001$). Furthermore, curcumin treatment increased the cellular NAD^+/NADH ratio in Gas and Sol (Figure V-3 and V-4, F = 39.74 for Gas, 50.97 for Sol, $P < 0.001$) and no interaction between two factors (n.s.). However, curcumin treatment increased the expression of the SIRT1 protein only in Gas (Figure V-5, F = 14.32, $P < 0.001$) but was not showed in Sol (Figure V-6, n.s.) and eTR increased SIRT1 protein expression in both muscle types (Gas and Sol) (Figure V-5 and V-6, F = 75.97 for Gas, 53.65 for Sol, $P < 0.001$) and no interaction between two factors (n.s.). Meanwhile, post-hoc analysis showed that both doses of curcumin significant different than DMSO to increase NAD^+/NADH ratio in both skeletal muscle (Figure V-3 and V-4, $P < 0.05$). On the other hand, only high dose curcumin showed different than DMSO to increase SIRT1 protein expression in Gas (Figure V-5, $P < 0.05$). These results indicate that high dose curcumin increases the expression of SIRT1 in skeletal muscle via an increase in the cellular NAD^+/NADH ratio and that this is additive to the effect of eTR.

C. Curcumin Treatment with eTR Decrease Acetylation of PGC-1α

We therefore examined the deacetylation of PGC-1α in skeletal muscle. As shown in Figure V-7 and V-8, two way ANOVA determined that curcumin treatment, decreased the acetylation of PGC-1α in Gas and Sol (F = 26.03, 14.09 respectively, $P < 0.001$). However eTR decreased the acetylation of PGC-1α in both Gas and Sol (Figure V-7 and V-8, F = 148.82 for Gas, 217.923 for Sol, P <

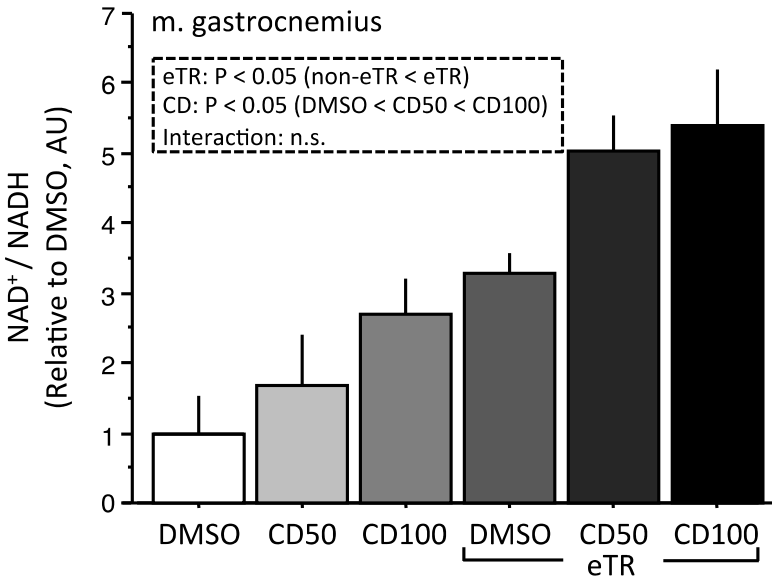


Figure V-3. Effect of curcumin treatment increases NAD^+/NADH ratio in gastrocnemius.

Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for NAD^+/NADH ratio in Gas, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increase NAD^+/NADH ratio in gastrocnemius ($P < 0.05$)

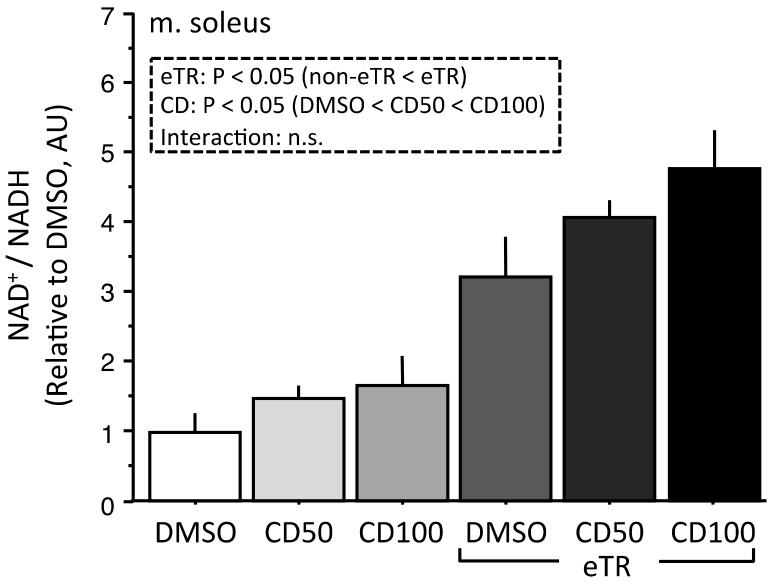


Figure V-4. Effect of curcumin treatment increases NAD^+/NADH ratio in soleus.

Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for NAD^+/NADH ratio in Sol, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin increase NAD^+/NADH ratio in soleus ($P < 0.05$)

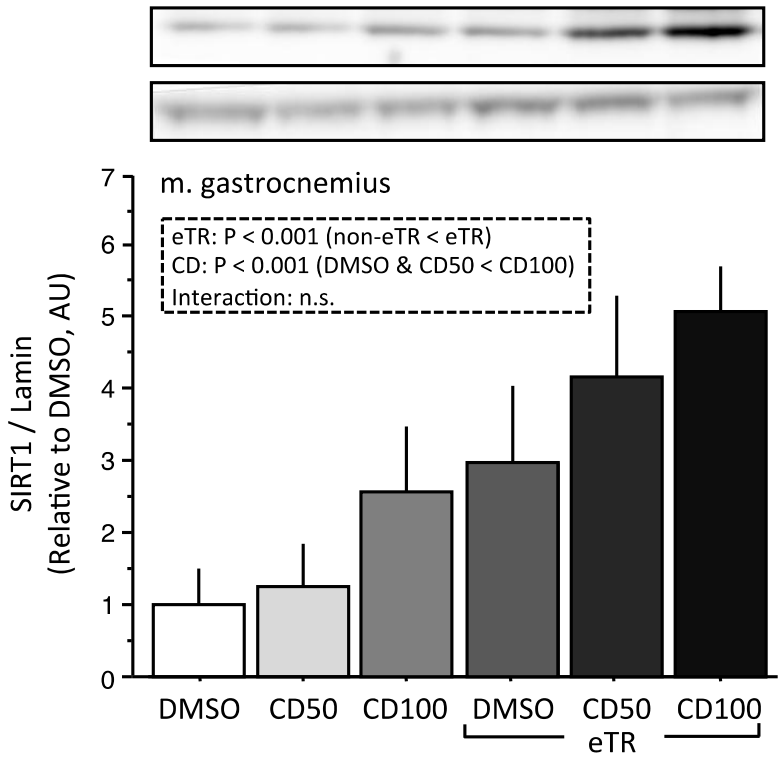


Figure V-5. Effect of curcumin treatment increases SIRT1 expression in gastrocnemius.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for SIRT1 expression in Gas, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, only high dose curcumin increase SIRT1 expression in gastrocnemius ($P < 0.05$)

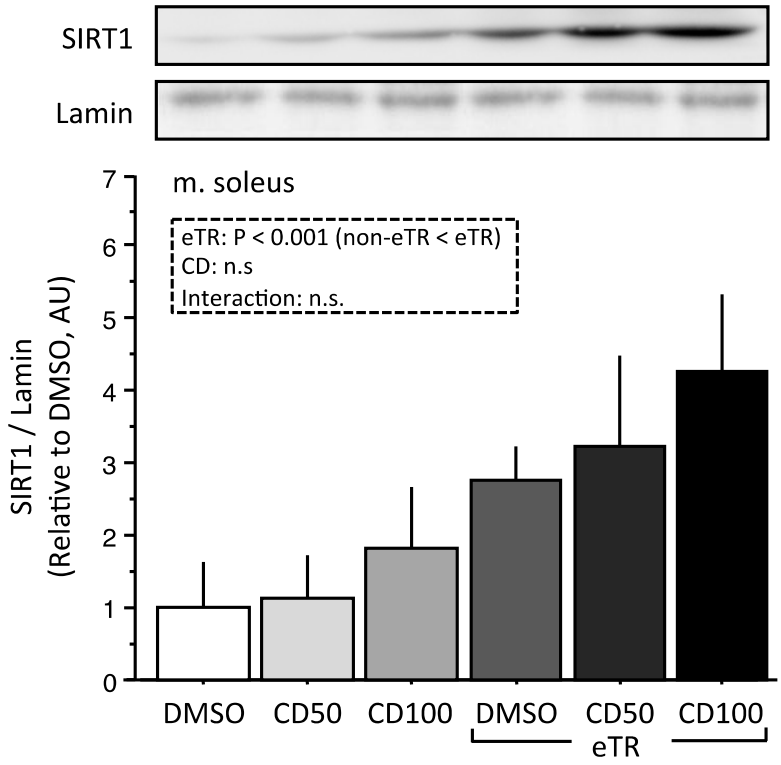


Figure V-6. Effect of curcumin treatment increases SIRT1 expression in soleus.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) for SIRT1 expression but was not showed for main effect of curcumin (n.s.).

0.001) and no interaction between two factor (n.s.). Furthermore, post-hoc analysis showed that low dose and high dose curcumin significant different than DMSO in Gas and Sol (Figure V-7 and V-8, $P < 0.05$) but was not different between low dose and high dose treatment (n.s.). This result together indicating that curcumin induced PGC-1α deacetylation in skeletal muscles in an additive manner to the effect produced by eTR.

4. Discussion

AMPK is a critical regulator of mitochondrial biogenesis in response to energy deprivation and previous studies have reported that its expression increases after exercise (Sakamoto et al., 2005; Stein et al., 2000). Furthermore, previous studies found that curcumin increases the phosphorylation of AMPK in skeletal muscle (Na et al., 2011). NAD^+ and NADH, are coenzymes required for the generation of ATP by mitochondria. SIRT1 is dependent on NAD^+ as a substrate and has therefore been proposed as a key regulator of the adaptation to acute and chronic exercise in the mitochondria of skeletal muscle (Gurd, 2011). Activation of PGC-1α is important both for the determination of mitochondrial content and the induction of mitochondrial biogenesis in skeletal muscle. Previous research has demonstrated that deacetylation of PGC-1α by SIRT1 is important for skeletal muscle function and the induction of mitochondrial biogenesis in response to exercise (Gurd, 2011).

Chronic exercise induces mitochondrial biogenesis together with changes in

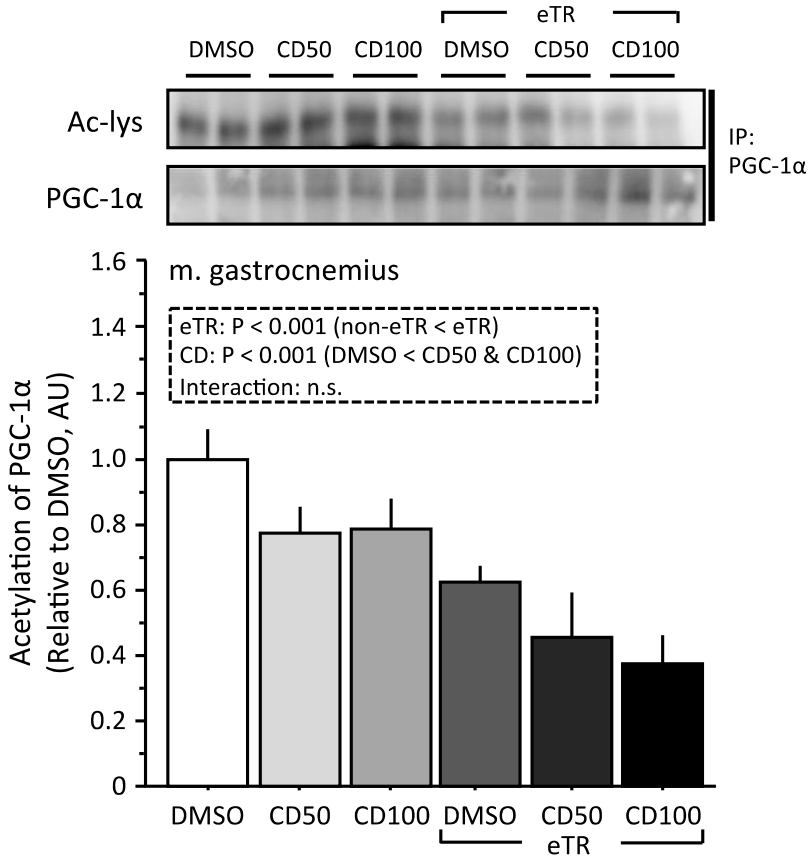


Figure V-7. Effect of curcumin treatment decreases acetylation of PGC-1α in gastrocnemius.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for decrease acetylation of PGC-1α in Gas, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin decrease acetylation of PGC-1α in gastronemius ($P < 0.05$)

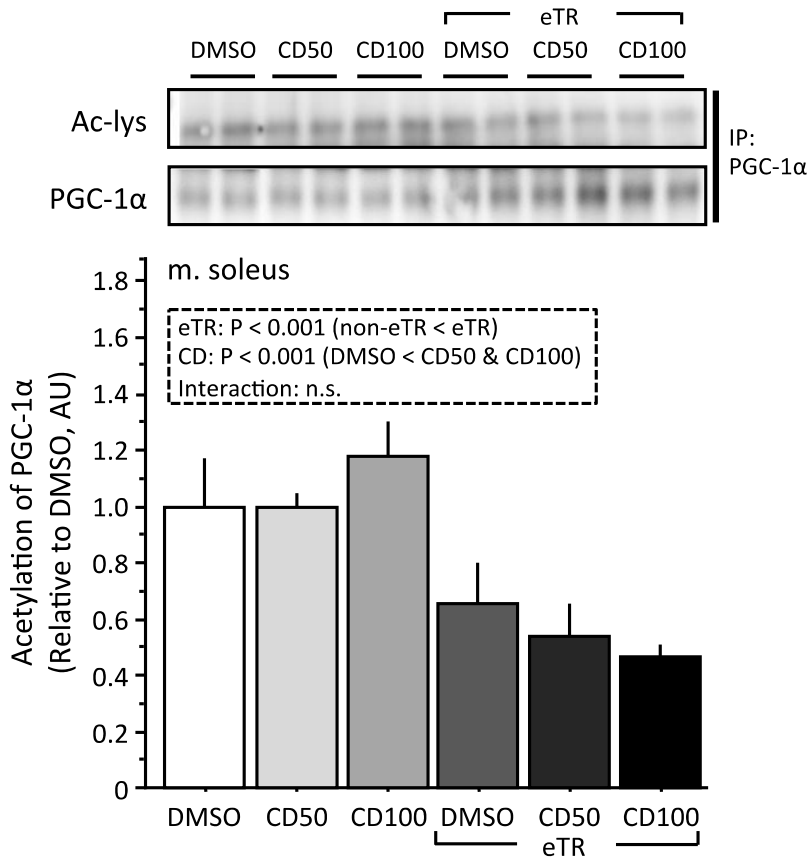


Figure V-8. Effect of curcumin treatment decreases acetylation of PGC-1α in soleus.

Value are mean ± SD (n = 6 in each group). DMSO = control. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) for decrease acetylation of PGC-1α in Sol, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses curcumin decrease acetylation of PGC-1α in soleus ($P < 0.05$)

indicators of insulin sensitivity and enhanced mitochondrial respiration in wild-type mice, which requires AMPK activation and may involve SIRT1-dependent PGC-1α deacetylation (Li et al., 2011). Similar with previous result our result showed evidence that 28 day swimming exercise increase mitochondrial biogenesis (result of experiment I) through increase phosphorylation AMPK, NAD+/NADH ratio, SIRT1 expression and deacetylation of PGC-1α (result of experiment II) in skeletal muscle.

It has been reported that feeding mice polyphenol resveratrol activates AMPK and SIRT1 in skeletal muscle leading to deacetylation and activation of PGC-1α, increased mitochondrial biogenesis, and improved running endurance (Lagouge et al., 2006). Based on that study, we suggested that polyphenol curcumin capable induces mitochondrial biogenesis through AMPK-SIRT1-PGC1α pathway. Indeed, our result showed that curcumin additively endurance exercise induces phosphorylation AMPK, NAD+/NADH ratio, SIRT1 expression and deacetylation of PGC-1α.

5. Summary

This result suggests that curcumin activates AMPK phosphorylation in skeletal muscles and that the effect is in addition to the response to eTR. Furthermore, these results also indicate that curcumin increases the expression of SIRT1 in skeletal muscles via an increase in the cellular NAD^+/NADH ratio and that this is additive to the effect of eTR. Parallel with above result this present result indicating that curcumin induced PGC-1α deacetylation in skeletal muscles

in an additive manner to the effect produced by eTR alone. This result together suggested that curcumin treatment additively endurance exercise to induce mitochondrial biogenesis through AMPK-SIRT1-PGC-1α pathway.

VI. Effect of Curcumin Treatment on cAMP Level in skeletal muscle (Experiment III)

1. Purpose

The result of previous experiments (I and II) have showed evidence that curcumin itself increase and additively endurance exercise induces mitochondrial biogenesis through AMPK-SIRT1-PGC-1α pathway in rat skeletal muscle. However, the direct target of curcumin to increase mitochondrial biogenesis remains unknown. In both mammalian cells and yeast, the regulation of mitochondrial biogenesis clearly involves the cAMP signaling pathway. Indeed, it has been shown that treatment of human preadipocytes with forskolin, which leads to an overactivation of the cAMP pathway, increased the copy number of mitochondrial DNA (Bogacka et al., 2005). Furthermore, cAMP will quickly hydrolyze to 5'AMP by enzyme Phosphodiesterase (PDE). Previous studies have provided evidence that exercise, including swimming and running increase cAMP levels in skeletal muscle and myocardium (Palmer, 1988) and the mitochondrial biogenesis seen after exercise may be attributable to this increase.

The purpose of this present experiment to determine whether curcumin treatment have ability to increase cAMP which involve in regulated mitochondrial biogenesis manner examine curcumin treatment to cAMP level in m. gastrocnemius. Our hypothesis said that curcumin treatment increases cAMP levels in skeletal muscle.

2. Methods

A. Animals and Exercise Training

Ten-week-old male Wistar rats (body weight, 282–375 g) were used for the experiments. The animals were randomly divided into 4 groups (six rats every groups): control without eTR group, curcumin 100 mg/kg-BW/day without eTR, control with eTR group, and curcumin 100 mg/kg-BW/day with eTR. All animals were intraperitoneally injected once daily for three days with DMSO solution containing curcumin or the same volume of DMSO (vehicle alone).

The exercise training group rats swam 2 h/day in four 30-min bouts separated by 5 min of rest. After the first 30-min bout, a weight equal to 2% of body weight was tied to the body of the rats. The rats swam with the weight attached for the remaining three exercise bouts. All rats swam in a barrel filled to a depth of 50 cm. The swimming area was 190 cm^2/rat (Kawanaka et al., 1985). The rats performed the above swimming protocol once a day for three days.

B. cAMP Direct Immunoassay Kit

Levels of cAMP in cell extracts were measured with the cAMP Direct Immunoassay Kit (ab65355, Abcam, UK) following the manufacturer's instructions. Briefly, add 50 μl of the acetylated Standard cAMP and test samples from to the Protein G coated 96-well plate. This step following add 10 μl of the reconstituted cAMP antibody per well to the standard cAMP and sample wells except the well with 0_B pmol cAMP for background reading). Incubate for 1

hour at room temperature with gentle agitation.Wash 5 times with 200 µl 1X Assay Buffer each wash.Completely empty the wells by tapping the plateon a new papertowel after each wash. After making sure there is no liquid left behind, add 100 µl of HRP developer and develop for 1 hour at room temperature with agitation. Finally, stop the reaction by adding 100 µl of 1M HCl (not provided) to each well (sample color should change from blue to yellow) and read the plate at OD 450 nm by spectrophotometry (Infinite 200 PRO, Tecan, USA).

C. Statistical Analysis

Two-way analysis of variance (ANOVA) as used to assess the main effect of exercise (non-eTR vs. eTR groups) and curcumin (non-curcumin vs. curcumin groups) and the exercise x curcumin interaction. The Tukey-Kramer post-hoc test was used for analysis to identify of difference between low dose and high dose curcumin groups. All data were expressed as mean ± standard deviation (SD). The level of significance was established at $P < 0.05$.

3. Result

A. Curcumin Treatment and Exercise Increases cAMP level

We therefore hypothesized that curcumin by itself and in combination with exercise, would increase the levels of cAMP in skeletal muscle. Curcumin indeed increased cAMP levels (Figure. VI-1, $F = 81.78$ $P < 0.001$). Furthermore, exercise resulted increase in Gas compared to that by non exercise (Figure. VI-1,

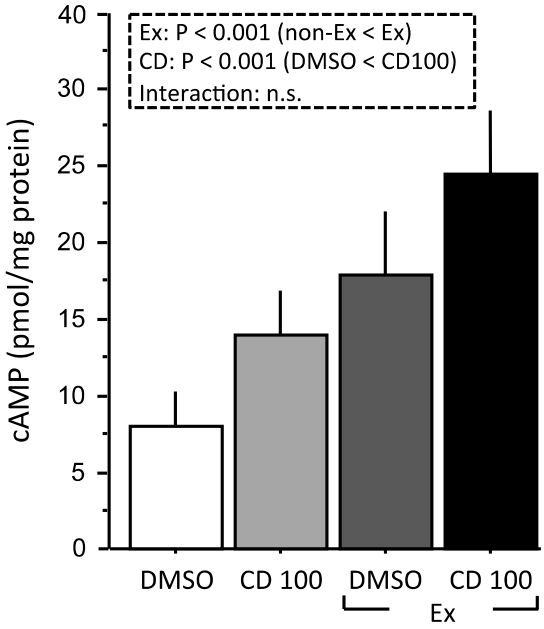


Figure VI-1. Effect of curcumin treatment increases cAMP level in gastrocnemius.

Value are mean ± SD (n = 6 in each group). DMSO = control. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. Ex = exercise. Two-way ANOVA revealed main effect for exercise ($P < 0.001$) and curcumin treatment ($P < 0.001$) for cAMP level in Gas, no interaction between two factor (n.s.). Post-hoc analysis revealed that compared to DMSO group, curcumin increase cAMP level in Gas ($P < 0.05$)

F = 211.26, P < 0.001) and no interaction between two factor (F = 0.29, P > 0.05).

4. Discussion

In both mammalian cells and yeast, the regulation of mitochondrial biogenesis clearly involves the cAMP (cyclic adenosine monophosphate) signaling pathway. Indeed, it has been shown that treatment of human preadipocytes with forskolin, which leads to an overactivation of the cAMP pathway, increased mitochondrial DNA copy number (Bogacka et al., 2005). The cAMP generated by adenylyl cyclases is tightly regulated by phosphodiesterases (PDE), which catabolize cAMP into 5′ AMP. It is well known that exercise increases the levels of cAMP (Ohyama et al, 2015) and curcumin has also been reported to have this effect as measured by inhibition of endothelial cell phosphodiesterase (PDE) (Abusnina et al, 2009). Our result showed that curcumin treatment (100 mg/kg-BW/day) increases cAMP level on gastrocnemius and when curcumin combined with exercise it seem look like that curcumin additively exercise effect to induce cAMP level in skeletal muscle.

5. Summary

This result suggested that curcumin treatment have ability to increase cAMP levels in skeletal muscle.

VII. Effect of Curcumin Treatment on cAMP – PKA pathway

1. Purpose

Protein kinase A (PKA), a well-studied cAMP downstream effector is also known as cAMP-dependent enzyme because it gets activated only if cAMP is present. Once PKA is activated, it phosphorylates a number of other proteins including cAMP response element binding protein (CREB) (Than et al., 2011) and liver kinase B1 (LKB1) (Veeranki et al., 2011) which induction PGC-1α to regulated mitochondria biogenesis. Previous study showed that PKA inhibitor (H89) inhibited mitochondrial biogenesis in brain which indicated that PKA-CREB plays a critical role in mitochondrial biogenesis in brain (Sheng et al., 2012).

The purpose of this present experiment to examine the downstream target of PKA included phosphorylation CREB and LKB-1 to determine whether cAMP needed in curcumin treatment induces mitochondrial biogenesis in skeletal muscle by used PKA inhibitor (H89). Our hypothesis suggested that curcumin increase the downstream target of PKA included phosphorylation CREB and LKB-1 and PKA inhibitor (H89) abolished this effect.

2. Methods

A. Animals

Ten-week-old male Wistar rats (body weight, 282–375 g) were used for the experiments. The animals were randomly divided into 6 groups: control

(DSMSO only) without exercise group, curcumin 100 mg/kg-BW/day without exercise, H89 (PKA inhibitor) 20 mg/kg-BW/ day without exercise, H89 + curcumin 100 mg/kg-BW/day without exercise, control (DMSO only) with exercise group, and curcumin 100 mg/kg-BW/day with exercise, H89 (PKA inhibitor) 20 mg/kg-BW/ day with exercise, H89 + curcumin 100 mg/kg-BW/day with exercise. All animals were intraperitoneally injected once daily for three days with DMSO solution containing curcumin, H89 or the same volume of DMSO (vehicle alone). Meanwhile, Exercise training protocol same with previous methods in this present study.

B. Western Blotting

Western blotting analysis was performed as previously described (Furuichi et al, 2010). Briefly, equal protein amounts of samples were loaded onto SDS-PAGE (SDS-polyacrylamide gel electrophoresis) gels 10% (p-LKB-1, t-LKB-1, p-CREB, t-CREB) and the proteins were transferred onto a polyvinylidene difluoride membrane (PVDF; polyvinylidene difluoride membrane, ATTO, Japan). The membrane was incubated in Tris-buffered saline (TBS) buffer (150 mm NaCl, 25 mm Tris-HCl, pH 7.4) containing 0.1% Tween-20 for 10 min at room temperature. The membrane was then incubated on 4% blocking buffer on room temperature for 1 hour. The membrane was then incubated with Rabbit polyclonal to Phospho LKB-1 (S 428) (1:500 dilution, #: ab63473-GR91843-2, Abcam, Cambridge, England), Rabbit polyclonal to LKB-1 (1:500 dilution, #: ab58786-GR146310-3, Abcam, Cambridge, England), Mouse monoclonal to

CREB (Phospho S133) (1:500 dilution, #: ab173780-GR316230-2, Abcam, Cambridge, England), Rabbit polyclonal to CREB + CREM (1:500 dilution, #: ab5803-GR156230-3, Abcam, Cambridge, England), Mouse monoclonal antibody to β-actin (1:1000 dilution, #: ab8226-GR110198-3, Abcam, Cambridge, England), Mouse monoclonal to GAPDH (1:1000 dilution, #: ab8245-GR168547-1, Abcam, Cambridge, England), and Rabbit polyclonal antibody to lamin A/C (1:1000 dilution, #: SC-20681-D0142, Santa Cruz Biotechnology, CA, USA) antibodies at 4°C for overnight and rinsed with TBS-T three times. It was then reacted with HRP (HRP; horseradish peroxidase-conjugated) Anti-rabbit IgG, HRP-linked Whole Ab donkey (Peroxidase-Linked Secondary Antibodies, GE healthcare, England) or Anti-mouse IgG HRP-linked Whole Ab donkey (Peroxidase-Linked Secondary Antibodies, GE healthcare, England) and signals were visualized by the enhanced chemiluminescence detection method using the ECL Plus Western blotting detection system (GE Healthcare, USA). The signal intensity was quantified using imaging software (Image J, version 1.46, USA).

C. Immunoprecipitation

The nuclear fraction was obtained using the same procedure as western blotting. In this preparation, buffer D (20 mM Tris-HCl [pH 7.4], 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM PMSF) was used instead of buffers A and C. The nuclear fraction of protein was divided

into 1-ml PBS aliquots. Dynabeads (Invitrogen, CA, USA) were added to the samples, and the samples were subsequently incubated at 4°C for 1 h. The Dynabeads were collected using magnets, and normal mouse immunoglobulin G (nIgG; Santa Cruz, USA) was added to the supernatants and incubated at 4°C overnight. Samples were centrifuged at 7000 *g* at 4°C for 30 s, and aliquots of this supernatant were reacted with primary antibodies against PGC-1α and mouse nIgG at 4°C overnight. Dynabeads were then added and incubated at 4°C for 2 h. The pellet was collected using magnets and washed with PBS. The final pellet from the immunoprecipitation (IP) was analyzed using SDS-PAGE and immunoblotting with western blotting method.

D. Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Variables among groups were compared using one-way ANOVA, and a Tukey-Kramer post-hoc test was performed if the ANOVA indicated a significant difference. The level of significance was established at $P < 0.05$.

3. Result

A. Curcumin Treatment and Exercise Increase Phosphorylation LKB-1

We determined the downstream target proteins of cAMP-dependent protein kinase **(**PKA) by using the PKA inhibitor H89. Our results showed that curcumin increased the phosphorylation of LKB-1 in group without exercise in Gas (Figure VII-1, 1,6 ± 0.85 for curcumin without exercise vs. 1.0 ± 0.26 for

DMSO without exercise, $P < 0.05$) and H89 abolished this effect (Figure VII-1, 0.78 ± 0.2, $P < 0.05$). Furthermore, curcumin augments exercise effect to increases phosphorylation of LKB-1 in Gas (Figure VII-1, 2.85 ± 0.22 for curcumin with exercise vs. 2.39 ± 0.45 for DMSO with exercise, $P < 0.05$) and H89 abolished this effect (Figure VII-1, 1.17 ± 0.54, $P < 0.05$). These results suggested that curcumin increases the phosphorylation of LKB1 in skeletal muscle in addition to the effect of exercise.

B. Curcumin Treatment and Exercise Increase Phosphorylation of CREB

In order to determine the parallel downstream target proteins of cAMP-dependent protein kinase **(**PKA). We examine effect curcumin and exercise to increase phosphorylation of cAMP response element binding protein (CREB) by using the PKA inhibitor H89. Our results showed that curcumin increased the phosphorylation of CREB in group without exercise in Gas (Figure VII-2, 1.37 ± 0.91 for curcumin without exercise vs. 1.0 ± 0.13 for DMSO without exercise, $P < 0.05$) and H89 abolished this effect (Figure VII-2, 0.44 ± 0.7, $P < 0.05$). Furthermore, curcumin augments exercise effect to increase phosphorylation of CREB in Gas (Figure VII-2, 2.11 ± 0.22 for curcumin with exercise vs. 1.76 ± 0.67 for DMSO with exercise, $P < 0.05$) and H89 abolished this effect (Figure VII-2, 0.56 ± 0.46, $P < 0.05$). These results suggested that curcumin increases the

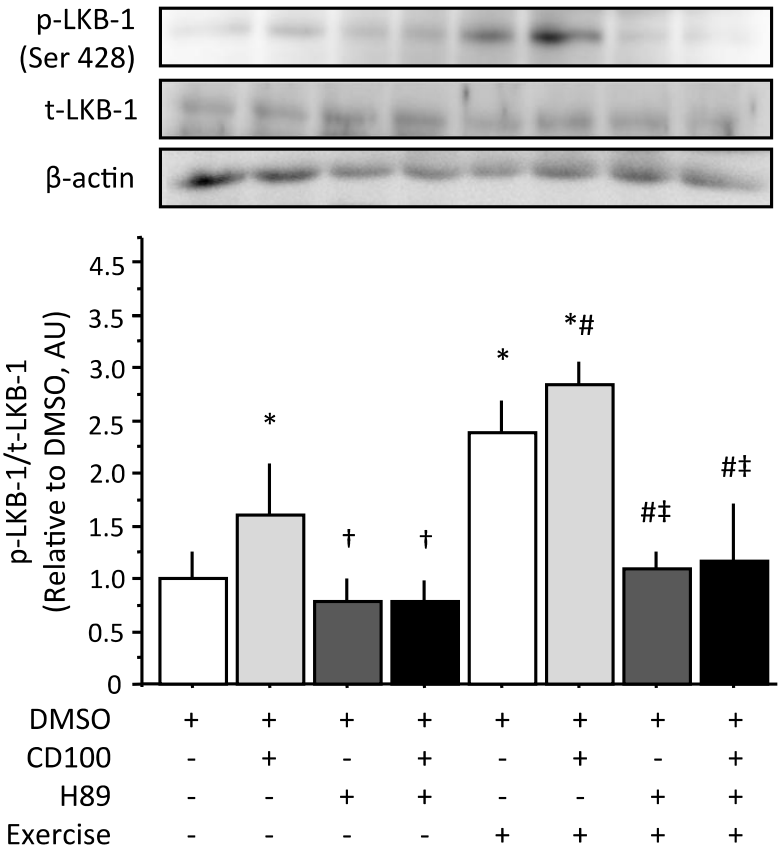


Figure VII-1. Effect of curcumin treatment increases phosphorylation of LKB-1 (Ser 428) in Gastrocnemius.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. *: significant difference from DMSO without exercise group ($P < 0.05$). #: significant difference from DMSO + exercise group ($P < 0.05$). †: significant different from curcumin without exercise ($P < 0.05$). ‡: significant different from curcumin + exercise.

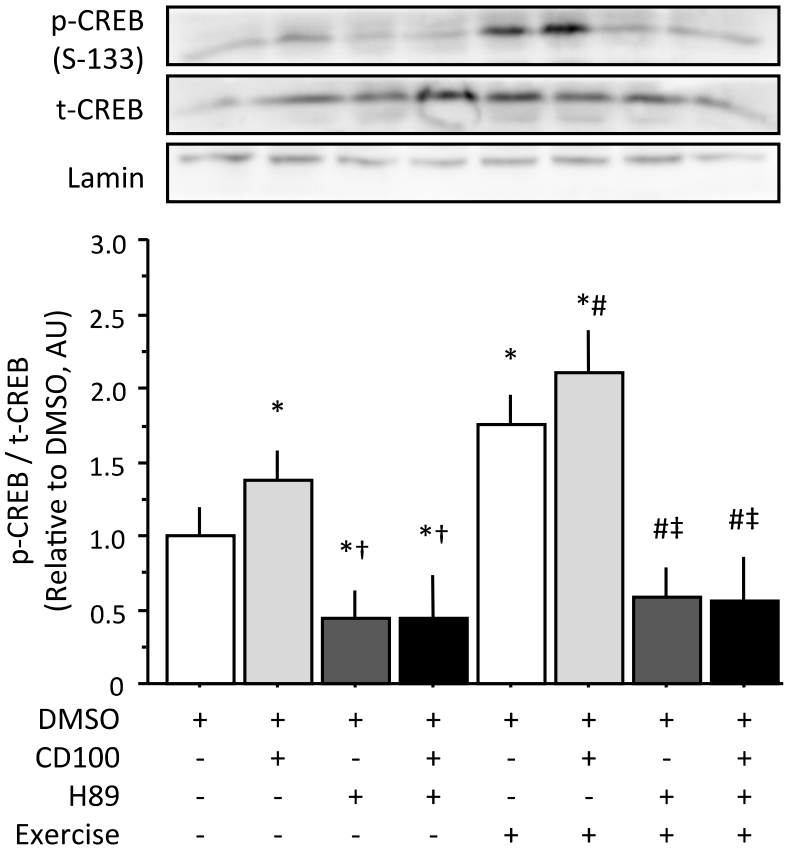


Figure VII-2. Effect of curcumin treatment increases phosphorylation of CREB (S-133) in Gastrocnemius.
Value are mean ± SD (n = 6 in each group). DMSO = control. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. *: significant difference from DMSO without exercise group ($P < 0.05$). #: significant difference from DMSO + exercise group ($P < 0.05$). †: significant different from curcumin without exercise ($P < 0.05$). ‡: significant different from curcumin + exercise.

the phosphorylation of CREB in skeletal muscle in addition to the effect of exercise.

C. Curcumin Treatment and Exercise Increase Phosphorylation of AMPK, COX-IV protein expression and Decrease Acetylation PGC-1α

We continuing to examine the downstream target from CREB and LKB-1 included phosphorylation AMPK, acetylation of PGC-1α and COX-IV expression in order to found out effect curcumin regulated mitochondrial biogenesis through cAMP - PKA pathway. Indeed, one-way ANOVA showed that curcumin together with exercise increases phosphorylation of AMPK in gas (Figure VII-3, 1.83 ± 0.24 for curcumin with exercise vs. 1.00 ± 0.94 for DMSO without exercise, $P < 0.05$) and H89 abolished this effect (Figure VII-3, 0.75 ± 0.12, $P < 0.05$). Addition, curcumin together with exercise also increases COX-IV expression (Figure VII-5, 2.4 ± 0.44 for curcumin with exercise vs. 1.00 ± 0.92 for DMSO without exercise, $P < 0.05$) and H89 abolished this effect (Figure VII-5, 0.97 ± 0.209, $P < 0.05$). Whether, curcumin together with exercise decrease acetylation of PGC-1α in Gas (Figure VII-4, 0.41 ± 0.53 for curcumin with exercise vs. 1.00 ± 0.26 for DMSO without exercise, $P < 0.05$).

4. Discussion

Protein kinase A (PKA), a well studied cAMP downstream effector is also known as cAMP-dependent enzyme because it gets activated only if cAMP is present. Once PKA is activated, it phosphorylates a number of other proteins

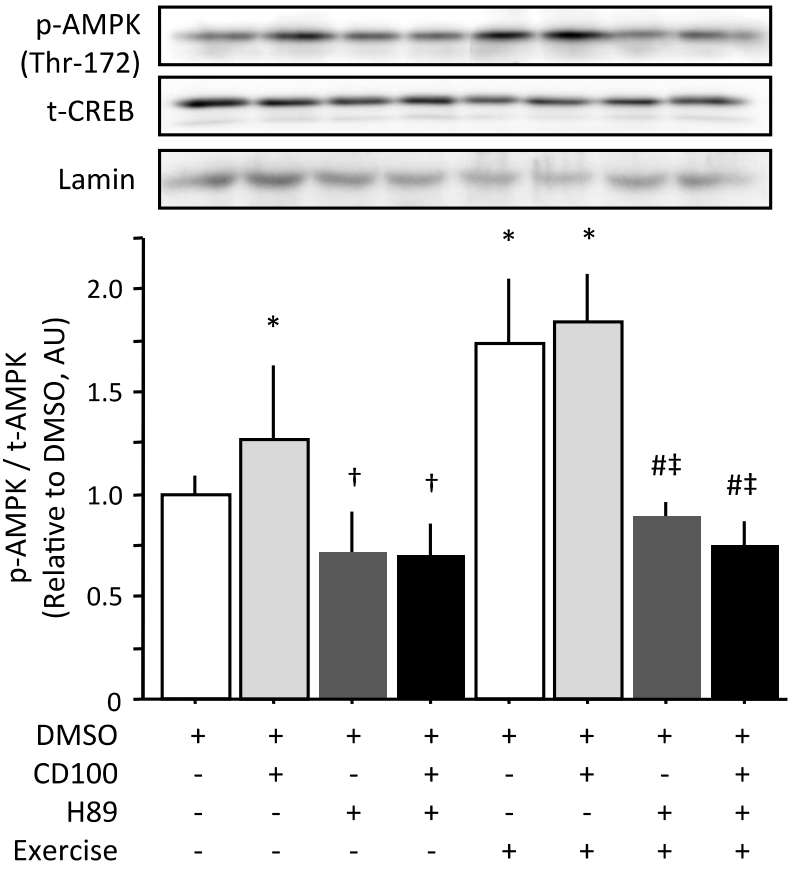


Figure VII-3. Effect of curcumin treatment increases phosphorylation of AMPK (Thr-172) in gastrocnemius.

Value are mean ± SD (n= 6 in each group). DMSO = control without exercise. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. *: significant difference from DMSO without exercise group ($P < 0.05$). #: significant difference from DMSO + exercise group ($P < 0.05$). †: significant different from curcumin without exercise ($P < 0.05$). ‡: significant different from curcumin + exercise.

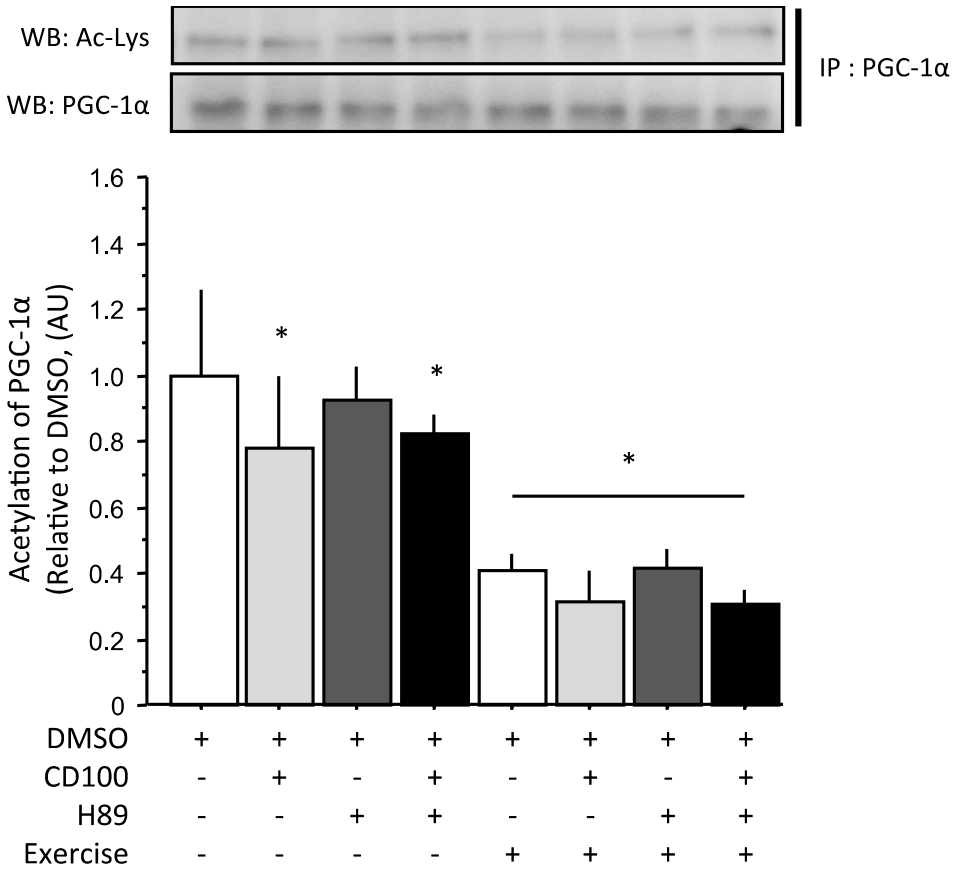


Figure VII-4. Effect of curcumin treatment decreases acetylation of PGC-1α in gastrocnemius.
Value are mean ± SD (n= 6 in each group). DMSO = control without exercise. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. *: significant difference from DMSO without exercise group ($P < 0.05$).

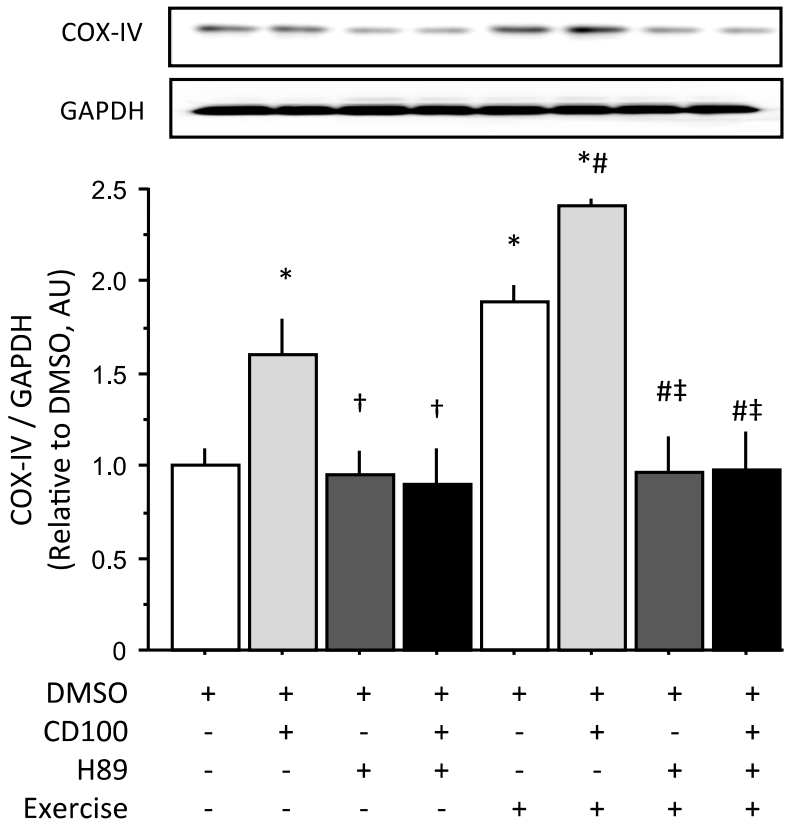


Figure VII-5. Effect of curcumin treatment increases COX-IV protein expression in gastrocnemius.

Value are mean ± SD (n= 6 in each group). DMSO = control without exercise. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. *: significant difference from DMSO without exercise group ($P < 0.05$). #: significant difference from DMSO + exercise group ($P < 0.05$). †: significant different from curcumin without exercise ($P < 0.05$). ‡: significant different from curcumin + exercise.

including cAMP response element binding protein (CREB) and liver kinase B1 (LKB-1) which induction PGC-1α to regulated mitochondria biogenesis (Than et al, 2011 and Veeranki et al, 2011). Previous study shown evidence that exercise included swimming and running increased cAMP on skeletal muscle and myocardium (Palmer, 1988). This evidence together strong suggested that exercise increased mitochondrial biogenesis through its ability to increase cAMP. Our result showed evidence that curcumin together with exercise increase phosphorylation LKB-1 and CREB. On the other hands, H89 abolished this effect. This result together indicates that cAMP is needed to regulate mitochondrial biogenesis in skeletal muscle and is in accordance with the results of a previous study, which showed that curcumin increased the phosphorylation of CREB in the brain (Nam et al., 2014) and of LKB-1 in skeletal muscle (Na et al., 2011). We continuing to examine downstream target from CREB and LKB-1 included phosphorylation of AMPK, acetylation of PGC-1α and COX-IV expression and indeed exercise and curcumin increase phosphorylation of AMPK, COX-IV expression and decrease acetylation of PGC-1α and H89 seem look like abolished this effect. Previous study showed evidence that LKB-1 induces phosphorylation of AMPK in skeletal muscle (Na et al., 2011) and following by induces PGC-1α to regulated mitochondria biogenesis. On the other hand, previous study has showed that CREB induces mitochondrial biogenesis through PGC-1α (Scarpulla, 2011).

5. Summary

These results suggest that curcumin increases the levels of cAMP as well as the phosphorylation of LKB-1, CREB, AMPK in skeletal muscle in addition to the effect of exercise.

VIII. Overall Discussion

The physiological significance of mitochondrial biogenesis is enhancement of cellular oxidative phosphorylation, which improves the endurance of the organism. Pathways that regulate mitochondrial biogenesis have recently emerged as potential metabolic targets. Previous studies presented evidence that curcumin could induce mitochondrial biogenesis in the liver and brain (Kuo et al., 2012a; Liu et al., 2014).

1. Curcumin Treatment and Endurance Exercise Increase Mitochondrial Marker in Skeletal Muscle

Our data support the finding that curcumin upregulates the expression of mitochondrial markers COX-IV (Figure IV-1 and IV-2), and OXPHOS sub unit (Figure IV-3 and IV-4) as well as increases the mtDNA copy number (Figure IV-5) and CS activity (Figure IV-6). Furthermore, we demonstrated that eTR increases in the expression of COX-IV (Figure IV-1 and IV-2), OXPHOS subunit (Figure IV-3 and IV-4), mitochondrial DNA copy number (Figure IV-5), and CS activity (Figure IV-6) as predicted. To our knowledge, this study is the first to demonstrate increased mitochondrial biogenesis in rat skeletal muscle stimulated by a combination of curcumin and exercise. The effect of curcumin appeared weaker in Sol than that in Gas and this maybe owing to differences in the pattern of recruitment of rat skeletal muscle during swimming (Roy et al., 1985). In addition, a previous study concluded that PGC-1α and mitochondria

content the fibers differ between species and that type IIa fibers, rather than type I fibers, are most represented in rodent skeletal muscle (Gouspillou et al., 2014). Previous research suggested that the polyphenol resveratrol combined with exercise could regulate mitochondria biogenesis through its antioxidant activity (Menzies et al., 2013). In fact, the increased mitochondrial capacity stimulated by physical exercise was actually ROS-dependent, and ROS generated in muscles during exhaustive activity was the stimulus that triggered mitochondrial biogenesis (Viña et al., 2009). Curcumin, however, is a more powerful antioxidant than other polyphenols including resveratrol (Aftab et al., 2010) and biomedical investigations of curcumin have provided evidence for a wide range of molecular and cellular activities, mostly related to redox reactions and signal transduction (Hatcher et al., 2008). We therefore speculated that the bioactivity of curcumin in skeletal muscles would be superior to that of resveratrol and directed through the regulation of mitochondrial biogenesis. In this study, curcumin treatment increased the levels of markers of mitochondrial biogenesis in skeletal muscle in a similar fashion to combination treatment with endurance training, suggesting that curcumin treatment together with exercise plays a role in the regulation of mitochondrial biogenesis in skeletal muscle.

2. Curcumin Treatment and Endurance Training Regulated Mitochondria Biogenesis in Skeletal Muscle Through AMPK-SIRT1-PGC-1α Mechanism Pathway

Exercise is known to induce mitochondrial biogenesis in skeletal muscles and improve performance by increasing oxidative capacity (Hood., 1985; Williams et al., 1986) for which the enzyme AMPK serves as a metabolic sensor. Structurally, mammalian AMPK is a heterotrimer of three subunits: one catalytic (α) and two regulatory (β and γ) subunits (Cheung et al., 2000; Stapleton et al., 1997). In skeletal muscles, exercise can elevate the cellular [AMP]/[ATP] ratio in cells (Chen et al., 2003), thereby activating AMPK (Hardie et al., 1998; Hardie et al., 2001; Zong et al., 2002), which is a critical regulator of mitochondrial biogenesis in response to energy deprivation (Zong et al., 2002). A previous study demonstrated that curcumin treatment could increase the phosphorylation of AMPK and alleviate insulin resistance in skeletal muscles (Na et al., 2011)**.** In adipocytes of C57/BL mice fed a high-fat diet, curcumin was shown to increase the phosphorylation of AMPK (Ejaz et al., 2009) suggesting that the AMPK pathway might be a target for curcumin in regulating mitochondrial biogenesis in skeletal muscles. Indeed, our results demonstrated that high doses of curcumin induced the phosphorylation of AMPK in Gas and Sol (Figure 4A, B). In addition, in accordance with the results of many recent studies, our results confirmed that eTR increased the phosphorylation of AMPK in skeletal muscles and furthermore that curcumin co-treatment additive the effect of eTR (Figure V-1 and V-2). When the intracellular energy state changes because of exercise, an increase in the cellular NAD^+ concentration and the resulting interaction of AMPK and SIRT1 can induce the expression of proteins involved in mitochondria biogenesis. In this study, curcumin was shown to

increase the [NAD^+] / [NADH] ratio (Fig . V-3 and V-4), resulting in an increase in SIRT1 expression (Figure V-5).

SIRT1-mediated PGC-1α deacetylation may be essential to activate mitochondrial biogenesis in skeletal muscles (Price et al., 2012; Rodgers et al., 2008). Thus, a number of studies have observed that SIRT1 levels of gene expression and protein increase in skeletal muscles in response to acute and chronic exercise, in parallel to an up-regulation of mitochondrial content. Our result demonstrated that curcumin treatment increased the cytosolic NAD^+/NADH ratio followed by an increase in the expression of SIRT1 protein in the nuclear fraction in Gas (Figure V-5). Furthermore, eTR also showed increased the NAD^+/NADH ratio and SIRT1 expression. In addition, some polyphenols, including curcumin, have also been shown to activate SIRT1 directly or indirectly in a variety of models (Chung et al., 2010; Queen et al., 2010). This has led to great interest in developing polyphenols to target the SIRT1-PGC-1α complex or related signaling pathways in order to regulate mitochondrial biogenesis in skeletal muscles, and mimic or potentiate the effect of eTR. One study has shown that the activation of SIRT1 by curcumin attenuates myocardial IR-induced mitochondrial oxidative damage (Yang et al., 2013). However, the present study is the first, to our knowledge, to demonstrate increases in the expression of SIRT1 protein in both skeletal muscle types in response to curcumin treatment combined with eTR (Figure IV-5).

PGC-1α is a coactivator, involved in activating both nuclear and mitochondrial transcription, resulting in mitochondrial biogenesis and the up-

regulation of genes involved in lipid metabolism and oxidative phosphorylation (Benton et al., 2008; Lin et al., 2002; Wu et al., 1999). Various transcriptional factors including nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (Tfam) may be involved (Kuo et al., 2012b). A previous study has shown that PGC-1α knockdown in skeletal muscle cells significantly reduced the expression of Tfam and COX-IV (Uguccioni et al., 2011). PGC-1α can also be regulated at the posttranslational level, for instance by acetylation (Rodgers et al., 2005). It has been reported that polyphenol combined with exercise increase deacetylation PGC-1α (Gurd, 2011). We therefore assessed the acetylation/deacetylation state of PGC-1α in the muscles of animals treated with curcumin with or without eTR. The ratio of acetylated PGC-1α to total PGC-1α protein in the nucleus was significantly decreased by curcumin treatment combined with eTR in both Gas and Sol (Figure V-7 and V-8) indicating that deacetylation of PGC-1α was facilitated by curcumin. Therefore, curcumin treatment had a significant impact on the state of PGC-1α and mitochondrial biogenesis when skeletal muscles were subjected to the repeated metabolic demands of eTR, and may have the potential to exert an additive effect together with eTR.

As discussed above, deacetylation of PGC-1α by SIRT1 is thought to be an important step in increasing PGC-1α activity, and SIRT1 protein expression increased with eTR and curcumin (Figure V-5). Although the mechanisms presently remain unclear, nitric oxide synthase (NOS) may be involved in this effect. Recent studies have shown that curcumin treatment increases NOS

enzyme activity in rat endothelium and increases the expression of eNOS and nNOS genes (Abdel et al., 2012; Nisoli et al., 2005). Another candidate is AMPK, since endurance exercise increases AMPK activity in skeletal muscles (Sakamoto et al., 2005; Stein et al., 2000). The results reported here regarding the increase in SIRT1 expression and NAD^+/NADH ratio in skeletal muscles following eTR in combination with curcumin treatment, may help explain the synergistic effects of the combined treatment *in vivo*. Our findings demonstrate the therapeutic potential of curcumin in the induction of mitochondrial biogenesis and function, which may be dependent on the presence of SIRT1 as well as on a cellular environment created by the continuous energy demands of repeated exercise. Similarly, the beneficial effects of both calorie restriction and the polyphenol resveratrol have been suggested to involve activation of SIRT1 and AMPK (Boily et al., 2009; Boily et al., 2008; Um et al., 2010). Thus, there is a dynamic interaction between these two pathways with AMPK activating SIRT1, probably through an indirect increase in cellular NAD^+ concentration (Canto et al., 2009). According to the above results, the present study suggests that the effects of phosphorylation and activation of AMPK (caused by exercise and curcumin treatment) trigger an increase in the cellular NAD^+/NADH ratio, which activates SIRT1. In addition, AMPK also phosphorylates PGC-1α and primes it for subsequent deacetylation by SIRT1. The effect of AMPK and SIRT1 on the acetylation status of PGC-1α, and possibly other transcriptional regulators, subsequently improves mitochondrial biogenesis (Figure VIII-1).

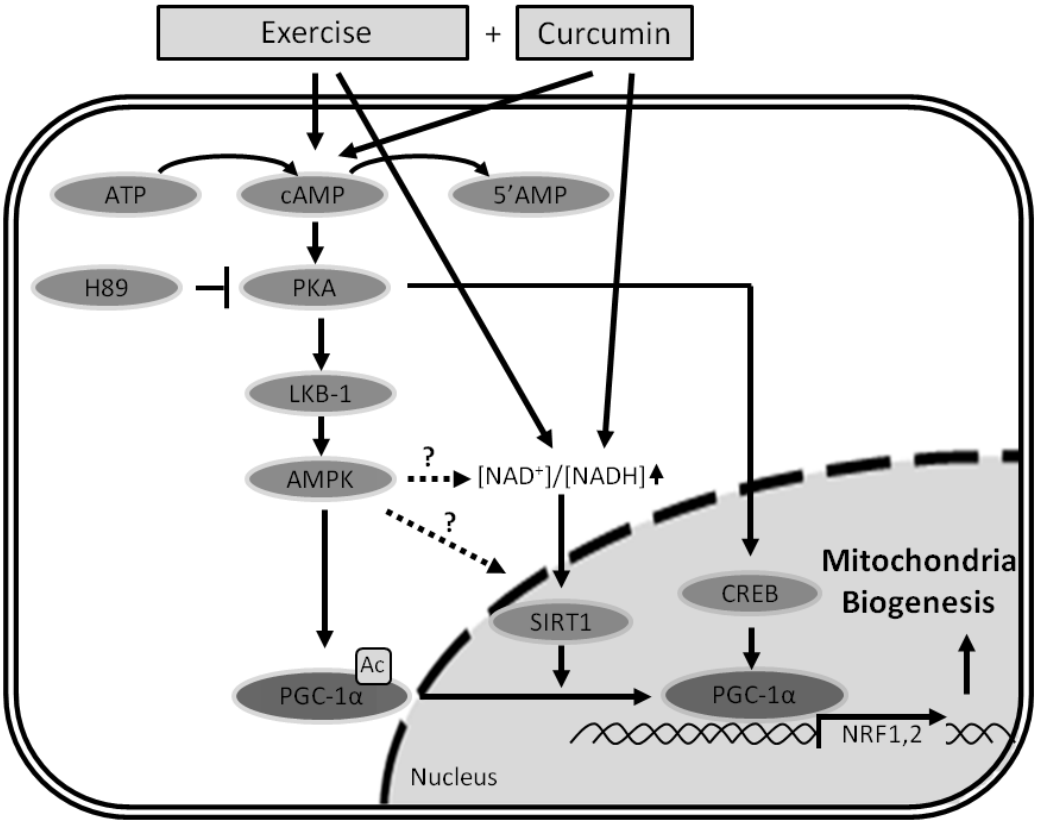


Figure VIII-1. Molecular pathways involved in the effects of exercise and curcumin treatment on the mitochondrial biogenesis.
Curcumin together with exercise increases cAMP level, which activates PKA and increase phosphorylation CREB and LKB-1. Phosphorylation and activation of AMPK triggers an increase in the cellular NAD^+/NADH ratio, which activates SIRT1. In addition, AMPK phosphorylates PGC-1α and primes it for subsequent deacetylation by SIRT1. The effect of AMPK and SIRT1 on the acetylation status of PGC-1α, and potentially of other transcriptional regulators, subsequently improves mitochondrial biogenesis. AMPK, 5' adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; eTR, endurance training; LKB-1, liver kinase B1; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrogen; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1; PKA, protein kinase A; SIRT1, sirtuin 1.

Since this is the first report showing the additive effect of curcumin and eTR on the phosphorylation AMPK in skeletal-muscle, the precise mechanism remains unknown.

3. Curcumin Treatment and Endurance Exercise Increase cAMP Level in Skeletal Muscle

cAMP is a critical, tightly regulated second messenger involved in many intracellular processes and degraded by PDE. cAMP activates PKA, which, in turn, phosphorylates CREB and LKB-1 thereby regulating mitochondrial biogenesis through PGC-1α. Our results indicated that curcumin and exercise increased the levels of cAMP in Gas (Fig 7A). This result confirms the results of a previous report, which demonstrated the potential of curcumin to increase cAMP levels (Abusnina et al., 2009; Rouse et al., 2014). Identification of cAMP as a target for curcumin in this present study might explain how curcumin mimics some aspects of exercise.

4. Curcumin Treatment and Exercise Increase Downstream Target of PKA

To determine whether cAMP is required to regulate mitochondrial biogenesis, we used PKA inhibitor H89 and showed that the induced phosphorylation of LKB-1 and CREB (Figure VII-1 and VII-2) in Gas was abolished (Figure VII-1 and VII-2). This result indicates that cAMP is needed to regulate mitochondrial biogenesis in skeletal muscle and is in accordance with the results of a previous study, which showed that curcumin increased the

phosphorylation of CREB in the brain (Nam et al., 2014) and of LKB-1 in skeletal muscle (Na et al., 2011b). Activated CREB and LKB-1 have proven to be important regulators of the PGC-1α promoter (Fernandez-Marcos et al., 2011; Koh et al, 2008). The expression of PGC-1α was mediated by the activation of the transcription factor CREB, which requires phosphorylation by PKA to initiate mitochondrial biogenesis (Kelly et al., 2004) while, LKB-1 regulated PGC-1α through the AMPK pathway (Koh et al., 2008). Indeed, our result showed that curcumin and exercise increase phosphorylation AMPK, decrease acetylation of PGC-1α and increase mitochondrial marker COX-IV expression. These results suggest that the ability to increase the levels of cAMP plays an important role in the adaptation of mitochondrial biogenesis by curcumin either alone or combined with exercise (Figure VIII-1). Since this the first study to report on the effect of curcumin together with exercise on skeletal muscle, further investigation will be necessary to describe the molecular mechanisms and identify whether curcumin directly induces AMPK and SIRT1 to regulate mitochondrial biogenesis in skeletal muscle.

IX. Summary

In the present study, we have investigated the mechanisms by which curcumin affects mitochondrial biogenesis in rats. We predicted that treatment with curcumin may have effects on the induction of mitochondrial biogenesis, and combined with endurance training (eTR), curcumin treatment addictively or synergistically enhances eTR-induced mitochondrial biogenesis. The primary purpose of the present study was to determine the effects of curcumin treatment combined with 24 days of eTR on regulation of mitochondrial biogenesis in skeletal muscles (m. gastrocnemius [Gas] and m. soleus [Sol]). The main findings from each experiment were as follows.

1. Effect of Curcumin Treatment on Mitochondrial Biogenesis Marker (Experiment I)

These experiment indicate that high-dose curcumin treatment and eTR additively increases the expression of mitochondria marker COX-IV and total OXPHOS subunit Complex I, II and III in skeletal muscle. Addition this experiment also has showed evidence that endurance training and curcumin treatment increase the mitochondrial copy number and CS activity in gastrocnemius and soleus muscle.

2. Mechanism Pathway How Curcumin Treatment Regulated Mitochondrial Biogenesis (Experiment II)

This result of this experiments suggests that curcumin activates AMPK phosphorylation in skeletal muscles and that the effect is in addition to the response to eTR. Furthermore, these results also indicate that curcumin increases the expression of SIRT1 in skeletal muscles via an increase in the cellular NAD^+/NADH ratio and that this is additive to the effect of eTR. Parallel with above result this present result indicating that curcumin induced PGC-1α deacetylation in skeletal muscles in an additive manner to the effect produced by eTR alone. This result together suggested that curcumin treatment additively endurance exercise to induce mitochondrial biogenesis through AMPK-SIRT1-PGC-1α pathway.

3. Effect of Curcumin Treatment on cAMP Level in skeletal muscle (Experiment III)

This experiment result suggested that curcumin treatment have ability to increase cAMP level in skeletal muscle.

4. Effect of Curcumin Treatment on cAMP-PKA Pathway (Experiment IV)

These experiment results suggest that curcumin increases the levels of cAMP as well as the phosphorylation of LKB-1, CREB and AMPK. Addition,

curcumin also decrease acetylation of PGC-1 and increase COX-IV expression in skeletal muscle in addition to the effect of exercise.

Acknowledgments

All praise, honour and glory to my Lord Jesus Christ for His richest grace and mercy for the accomplishment of this dissertation. I would never have been able to finish my dissertation without the guidance of my supervisor, help from friends, and support from my wife Evi Hadiany Isnaneningsih and my children (Darryl and Ranviel).

Over three years I have received support and encouragement from a great number of individuals. Prof. Kazumi MASUDA has been a mentor, colleague, and friend. His guidance has made this a thoughtful and rewarding journey. I would like to thank my collaborators Tatsuya Yamada, Ph.D, Rie Ishizawa, Ph.D, Yoko Saito, Ph.D for their support over the past two years as I moved from an idea to a completed study. In addition, all member of Kazumi Masuda laboratory for always supporting and encouraging me. Special thank for DIKTI who give scholarship and give me opportunity to study abroad and get the new experience which valuable for my future.

References

Abdel, A. M. T., Motawi, T., Rezq, A., Mostafa ,T., Fouad, H. H., Ahmed, H. H., Rashed, L., Sabry, D., Senbel, A., Al-Malki, A. and El-Shafiey, R. (2012) Effects of a water-soluble curcumin protein conjugate vs. pure curcumin in a diabetic model of erectile dysfunction. J. Sex. Med. 9: 1815-1833.

Abusnina, A., Keravis, T. and Lugnier, C. (2009) D020 The polyphenol curcumin inhibits in vitro angiogenesis and cyclic nucleotide phosphodiesterases (PDEs) activities similarly to PDE inhibitors. Arch. Cardiovasc. Dis. 102, Supplement 1: S43.

Adhihetty, P. J., Uguccioni, G., Leick, L., Hidalgo, J., Pilegaard, H. and Hood, D. A. (2009) The role of PGC-1alpha on mitochondrial function and apoptotic susceptibility in muscle. Am. J. Physiol. Cell. Physiol. 297: C217-225.

Aftab, N. and Vieira, A. (2010) Antioxidant activities of curcumin and combinations of this curcuminoid with other phytochemicals. Phytother. Res. 24: 500-502.

Akimoto, T., Sorg, B. S. and Yan, Z. (2004) Real-time imaging of peroxisome proliferator-activated receptor-γ coactivator-1α promoter activity in skeletal muscles of living mice, Am. J. Physiol. Cell. Physiol. 287: 790-796.

Alexander, A. and Walker, C. L. (2011) The role of LKB1 and AMPK in cellular responses to stress and damage. FEBS Letters 585: 952-957.

Aquilano, K., Vigilanza, P., Baldelli, S., Pagliei, B., Rotilio, G. and Ciriolo, M. R. (2010) Peroxisome proliferator-activated receptor gamma co-activator 1alpha (PGC-1alpha) and sirtuin 1 (SIRT1) reside in mitochondria: possible direct function in mitochondrial biogenesis. J. Biol. Chem. 285: 21590-21599.

Baar, K., Wende, A. R., Jones, T. E., Marison, M., Nolte, L. A., Chen, M., Kelly, D. P. and Holloszy, J.O (2002) Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. Faseb J 16: 1879-1886.

Bach, D., Pich, S., Soriano, F. X., Vega, N., Baumgartner, B., Oriola, J., Daugaard, J. R., Lloberas, J., Camps, M., Zierath, J. R., Rabasa-Lhoret, R., Wallberg-Henriksson, H., Laville, M., Palacin, M., Vidal, H., Rivera, F., Brand, M. and Zorzano, A. (2003) Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. J. Biol. Chem. 278: 17190-17197.

Bai, P., Canto, C., Oudart, H., Brunyanszki, A., Cen, Y., Thomas, C., Yamamoto, H., Huber, A., Kiss, B., Houtkooper, R. H., Schoonjans, K., Schreiber, V., Sauve, A. A., Menissier-de Murcia, J. and Auwerx, J. (2011) PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. Cell. Metab. 13: 461-468.

Baker, M. J., Frazier, A. E., Gulbis, J. M. and Ryan, M. T. (2007) Mitochondrial protein-import machinery: correlating structure with function. Trends. Cell. Biol. 17: 456-464.

Benton, C. R., Nickerson, J. G., Lally, J., Han, X. X., Holloway, G.P., Glatz, J. F., Luiken, J. J., Graham,T. E., Heikkila, J. J. and Bonen, A. (2008) Modest PGC-1alpha overexpression in muscle in vivo is sufficient to increase insulin sensitivity and palmitate oxidation in subsarcolemmal, not intermyofibrillar, mitochondria. J. Biol .Chem. 283: 4228-4240.

Bereiter-Hahn, J. (1990) Behavior of mitochondria in the living cell. Int. Rev. Cytol. 122: 1-63.

Bergeron, R., Ren, J. M., Cadman, K. S., Moore, I. K., Perret, P., Pypaert, M., Young, L. H., Semenkovich, C. F. and Shulman ,G. I. (2001) Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. Am. J. Physiol 281: E1340-1346.

Blough, E., Dineen, B. and Esser, K. (1999) Extraction of nuclear proteins from striated muscle tissue. BioTechniques 26: 202-204, 206.

Bogacka, I., Ukropcova, B., McNeil, M., Gimble, J. M. and Smith, S. R. (2005) Structural and functional consequences of mitochondrial biogenesis in human adipocytes in vitro. J. Clin. Endocrinol. Metab. 90: 6650-6656.

Boily, G., He, X. H., Pearce, B., Jardine, K. and McBurney, M. W. (2009) SirT1-null mice develop tumors at normal rates but are poorly protected by resveratrol. Oncogene 28: 2882-2893.

Boily, G., Seifert, E. L., Bevilacqua, L., He, X. H., Sabourin, G., Estey, C., Moffat, C., Crawford, S., Saliba, S., Jardine, K., Xuan, J., Evans, M., Harper, M. E. and McBurney, M. W. (2008) SirT1 regulates energy metabolism and response to caloric restriction in mice. PLoS One 3: e1759.

Cammarota, M., Paratcha, G., Bevilaqua, L. R., Levi de Stein, M., Lopez, M., Pellegrino de Iraldi, A., Izquierdo, I. and Medina, J. H. (1999) Cyclic AMP-responsive element binding protein in brain mitochondria. J. Neurochem. 72: 2272-2277.

Canto, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., Elliott, P. J., Puigserver, P. and Auwerx, J. (2009) AMPK regulates energy

expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 458: 1056-1060.

Canto, C., Pich, S., Paz, J. C., Sanches, R., Martinez, V., Orpinell, M., Palacin, M., Zorzano, A. and Guma, A. (2007) Neuregulins increase mitochondrial oxidative capacity and insulin sensitivity in skeletal muscle cells. Diabetes 56: 2185-2193.

Carling, D., Zammit, V. A. and Hardie, D. G. (1987) A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Letters 223: 217-222.

Carter, H.N., Chen, C. C. W. and Hood, D. A. (2015) Mitochondria, Muscle Health, and Exercise with Advancing Age. Physiol. 30: 208-223.

Chen, Z. P., Stephens, T. J., Murthy, S., Canny, B. J., Hargreaves, M., Witters, L. A., Kemp, B. E. and McConell, G. K. (2003) Effect of exercise intensity on skeletal muscle AMPK signaling in humans. Diabetes 52: 2205-2212.

Cheung, P. C., Salt, I. P., Davies, S. P., Hardie, D. G. and Carling, D. (2000) Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. Biochem J 346: 659-669.

Chin, E. R. (1985) Role of Ca^{2+}/calmodulin-dependent kinases in skeletal muscle plasticity. J. Appl. Physiol. 99: 414-423.

Chowanadisai, W., Bauerly, K. A., Tchaparian, E., Wong, A., Cortopassi, G. A. and Rucker, R. B. (2010) Pyrroloquinoline quinone stimulates mitochondrial biogenesis through cAMP response element-binding protein phosphorylation and increased PGC-1alpha expression. J. Biol. Chem. 285: 142-152.

Chung, S., Yao, H., Caito, S., Hwang, J. W., Arunachalam, G. and Rahman, I. (2010) Regulation of SIRT1 in cellular functions: role of polyphenols. Arch. Biochem. Biophys. 501: 79-90.

Conkright, M. D., Canettieri, G., Screaton, R., Guzman, E., Miraglia, L., Hogenesch, J. B. and Montminy, M. (2003) TORCs: transducers of regulated CREB activity. Mol. Cell. 12: 413-423.

Corton, J. M., Gillespie, J. G., Hawley, S. A. and Hardie, D. G. (1995) 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur. J. Biochem. 229: 558-565.

Dallas, C., Gerbi, A., Tenca, G., Juchaux, F. and Bernard, F. X. (2008) Lipolytic effect of a polyphenolic citrus dry extract of red orange, grapefruit, orange

(SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE). Phytomedicine 15: 783-792.

Dickinson, A., Yeung, K.Y., Donoghue, J., Baker, M. J., Kelly, R. D., McKenzie. M., Johns, T. G. and St. John, J. C. (2013) The regulation of mitochondrial DNA copy number in glioblastoma cells. Cell. Death. Differ. 20: 1644-1653.

Dillon, L. M., Rebelo, A. P. and Moraes, C. T. (2012) The role of PGC-1 coactivators in aging skeletal muscle and heart. IUBMB Life 64: 231-241.

Diviani, D., Dodge-Kafka, K. L., Li, J., Kapiloff, M. S. (2011) A-kinase anchoring proteins: scaffolding proteins in the heart. J. Physiol. Heart. Circ. Physiol. 301: 1742-1753.

Dorta, D. J., Pigoso, A. A., Mingatto, F. E., Rodrigues, T., Prado, I. M., Helena, A. F., Uyemura, S. A., Santos, A. C. and Curti, C. (2005) The interaction of flavonoids with mitochondria: effects on energetic processes. Chem. Biol. Interact. 152: 67-78.

Eckert, G. P., Schiborr, C, Hagl, S., Abdel-Kader, R., Muller, W. E., Rimbach, G. and Frank, J. (2013) Curcumin prevents mitochondrial dysfunction in the brain of the senescence-accelerated mouse-prone 8. Neurochemistry international 62: 595-602

Ejaz, A., Wu, D., Kwan, P. and Meydani, M. (2009) Curcumin Inhibits Adipogenesis in 3T3-L1 Adipocytes and Angiogenesis and Obesity in C57/BL Mice. The. J. Nut. 139: 919-925.

Fernandez-Marcos, P. J. and Auwerx, J. (2011) Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis. Am. J. Clin. Nutr. 93: 2.

Ferrara, N., Rinaldi, B., Corbi, G., Conti, V., Stiuso, P., Boccuti, S., Rengo, G., Rossi, F. and Filippelli, A. (2008) Exercise training promotes SIRT1 activity in aged rats. Rejuvenation Res. 11: 139-150.

Fisher, R. P. and Clayton, D. A. (1985) A transcription factor required for promoter recognition by human mitochondrial RNA polymerase. Accurate initiation at the heavy- and light-strand promoters dissected and reconstituted in vitro. J. Biol. Chem. 260: 11330-11338.

Furuichi, Y., Sugiura, T., Kato, Y., Shimada, Y. and Masuda, K. (2010) OCTN2 is associated with carnitine transport capacity of rat skeletal muscles. Acta Physiol. 200: 57-64.

Garcia-Roves, P. M., Osler, M. E., Holmstrom, M. H. and Zierath, J. R. (2008) Gain-of-function R225Q mutation in AMP-activated protein kinase

gamma3 subunit increases mitochondrial biogenesis in glycolytic skeletal muscle. J. Biol. Chem. 283: 35724-35734.

Garnier, A., Fortin, D., Zoll, J., N'Guessan, B., Mettauer, B., Lampert, E., Veksler, V. and Ventura-Clapier, R. (2005) Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. Faseb. J. 19: 43-52.

Gerhart-Hines, Z., Rodgers, J. T., Bare, O., Lerin, C., Kim, S. H., Mostoslavsky, R., Alt, F. W., Wu, Z. and Puigserver, P. (2007) Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. Embo. J. 26: 1913-1923.

Girija, C. R., Karunakar, P., Poojari C. S., Begum, N. S. and Syed, A. A. Molecular Docking Studies of Curcumin Derivatives with Multiple Protein Targets for Procarcinogen Activating Enzyme Inhibition. 2010. J Proteomics Bioinform 3: 200- 203.

Gledhill, J. R., Montgomery, M. G., Leslie, A. G. and Walker, J. E. (2007) Mechanism of inhibition of bovine F1-ATPase by resveratrol and related polyphenols. Proc. Natl. Acad. Sci. U. S. A. 104: 13632-13637.

Goldberg, D. M., Yan, J. and Soleas, G. J. (2003) Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin. Biochem. 36: 79-87.

Gollnick, P. D., Armstrong, R. B., Saubert, C, W., Piehl, K. and Saltin, B. (1972) Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. J. Appl. Physiol. 33: 312-319.

Gouspillou, G., Sgarioto, N., Norris, B., Barbat-Artigas, S., Aubertin-Leheudre, M., Morais, J.A., Burelle, Y., Taivassalo, T. and Hepple, R. T. (2014) The relationship between muscle fiber type-specific PGC-1alpha content and mitochondrial content varies between rodent models and humans. PLoS One 9.

Grubisha, O., Smith, B. C. and Denu, J. M. (2005) Small molecule regulation of Sir2 protein deacetylases. Febs. J. 272: 4607-4616.

Gurd, B. J. (2011) Deacetylation of PGC-1alpha by SIRT1: importance for skeletal muscle function and exercise-induced mitochondrial biogenesis. Appl. Physiol. Nutr. Metab. 36: 589-597.

Handschin, C., Rhee, J., Lin, J., Tarr, P. T. and Spiegelman, B. M. (2003) An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. Proc. Natl. Acad. Sci. U. S. A. 100: 7111-7116.

Hardie, D. G. (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat. Rev. Mol. Cell. Biol. 8: 774-785.

Hardie, D. G., Carling, D. and Carlson, M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu. Rev. Biochem. 67: 821-855.

Hardie, D. G. and Hawley, S. A. (2001) AMP-activated protein kinase: the energy charge hypothesis revisited. BioEssays 23: 1112-1119.

Hariharan, N., Maejima, Y., Nakae, J., Paik, J., Depinho, R. A. and Sadoshima, J. (2010) Deacetylation of FoxO by Sirt1 Plays an Essential Role in Mediating Starvation-Induced Autophagy in Cardiac Myocytes. Circ. Res. 107: 1470-1482.

Hatcher, H., Planalp, R., Cho, J., Torti, F. M. and Torti, S. V. (2008) Curcumin: from ancient medicine to current clinical trials. Cell. Mol. Life. Sci. 65: 1631-1652.

Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R. and Hardie, D. G. (2003) Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J. Biol. 2: 24.

Hodnick, W. F., Bohmont, C. W., Capps, C. and Pardini, R. S. (1987) Inhibition of the mitochondrial NADH-oxidase (NADH-coenzyme Q oxido-reductase) enzyme system by flavonoids: a structure-activity study. Biochem. Pharmacol. 36: 2873-2874.

Hodnick, W. F., Kung, F. S., Roettger, W. J., Bohmont, C. W. and Pardini, R. S. (1986) Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. A structure-activity study. Biochem. Pharmacol. 35: 2345-2357.

Holloszy, J. O. (1967) Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. J. Biol. Chem. 242: 2278-2282.

Holloszy, J. O. (2008) Regulation by exercise of skeletal muscle content of mitochondria and GLUT4. J. Physiol. Pharmacol. 59 Suppl 7: 5-18.

Holloszy, J. O, Oscai, L. B., Don, I. J. and Molé, P. A. (1970) Mitochondrial citric acid cycle and related enzymes: Adaptive response to exercise. Biochem. Biophysic. Res. Com. 40: 1368-1373.

Hood, D. A. (1985) Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. J. Appl. Physiol. 90: 1137-1157.

Hood, D. A., Adhihetty, P. J., Colavecchia, M., Gordon, J. W., Irrcher, I., Joseph, A. M., Lowe, S. T. and Rungi, A. A. (2003) Mitochondrial biogenesis and the role of the protein import pathway. Med. Sci. Sports. Exerc. 35: 86-94.

Hood, D. A., Saleem, A. (2007) Exercise-induced mitochondrial biogenesis in skeletal muscle. Nutr. Metab. Cardiovasc. Dis. 17: 332-337.

Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Hanafusa, T. and Matsuzawa, Y. (2000) Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler. Thromb. Vasc. Biol. 20: 1595-1599.

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W, Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L. L., Scherer, B. and Sinclair, D. A. (2003) Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature 425: 191-196.

Irrcher, I., Adhihetty, P. J., Sheehan, T., Joseph, A. M. and Hood, D. A. (2003) PPARgamma coactivator-1alpha expression during thyroid hormone- and contractile activity-induced mitochondrial adaptations. Am. J. Physiol. Cell. Physiol. 284: C1669-1677.

Jeppesen, J., Maarbjerg, S. J., Jordy, A. B., Fritzen, A. M., Pehmoller, C., Sylow, L., Serup, A. K., Jessen, N., Thorsen, K., Prats, C., Qvortrup, K., Dyck, J. R., Hunter, R. W., Sakamoto, K., Thomson, D. M., Schjerling, P., Wojtaszewski, J. F., Richter, E. A. and Kiens, B. (2013) LKB1 regulates lipid oxidation during exercise independently of AMPK. Diabetes 62: 1490-1499.

Jorgensen, S. B, Treebak, J. T, Viollet, B., Schjerling, P., Vaulont, S., Wojtaszewski, J. F. and Richter, E. A. (2007) Role of AMPKalpha2 in basal, training-, and AICAR-induced GLUT4, hexokinase II, and mitochondrial protein expression in mouse muscle. Am. J. Physiol. 292: 331-339,.

Joseph, A. M., Joanisse, D. R., Baillot, R. G. and Hood, D. A. (2012) Mitochondrial dysregulation in the pathogenesis of diabetes: potential for mitochondrial biogenesis-mediated interventions. Exp. Diabetes. Res. 2012: 642038.

Jornayvaz, F. R. and Shulman, G. I. Regulation of mitochondrial biogenesis. (2010). Essays Biochem. 2010: 47:69-84.

Kaeberlein, M., McDonagh, T., Heltweg, B., Hixon, J., Westman, E. A., Caldwell, S. D., Napper, A., Curtis, R., DiStefano, P. S., Fields, S.,

Bedalov, A. and Kennedy, B. K. (2005) Substrate-specific activation of sirtuins by resveratrol. J. Biol. Chem. 280: 17038-17045.

Kawanaka, K., Tabata, I., Katsuta, S. and Higuchi, M. (1985) Changes in insulin-stimulated glucose transport and GLUT-4 protein in rat skeletal muscle after training. J. Appl. Physiol. 83: 2043-2047.

Kelly, D. P. and Scarpulla, R. C. (2004) Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes. Dev. 18: 357-368.

Kidd, P. M. (2009) Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. Altern. Med. Rev. 14: 226-246.

Kim, C., Xuong, N. H. and Taylor, S. S. (2005) Crystal structure of a complex between the catalytic and regulatory (RI alpha) subunits of PKA. Science 307: 690-696.

Koh, H. J., Brandauer, J. and Goodyear, L. J. (2008) LKB1 and AMPK and the regulation of skeletal muscle metabolism. Curr. Opin. Clin. Nutr. Metab. Care. 11: 227-232.

Kuo, J. J., Chang, H. H., Tsai, T. H. and Lee, T. Y. (2012a) Curcumin ameliorates mitochondrial dysfunction associated with inhibition of gluconeogenesis in free fatty acid-mediated hepatic lipoapoptosis. Int. J. Mol. Med. 30: 643-649.

Kuo, J. J., Chang, H. H., Tsai, T. H. and Lee, T. Y. (2012b) Positive effect of curcumin on inflammation and mitochondrial dysfunction in obese mice with liver steatosis. Int. J. Mol. Med. 30: 673-679.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., Geny, B., Laakso, M., Puigserver, P. and Auwerx, J. (2006) Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell 127: 1109-1122.

Larsson, N. G., Oldfors, A., Holme, E. and Clayton, D. A. (1994) Low levels of mitochondrial transcription factor A in mitochondrial DNA depletion. Biochem. Biophysc. Res. Com. 200: 1374-1381.

Larsson, N. G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S., Clayton, D. A. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat. Genet. 18: 231-236.

Lee, I. H., Cao, L., Mostoslavsky, R., Lombard, D. B., Liu, J., Bruns, N. E., Tsokos, M., Alt, F. W. and Finkel, T. (2008) A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. Proc. Natl. Acad. Sci. U. S. A. 105: 3374-3379.

Lee, J., Kim, C. H., Simon, D. K., Aminova, L. R., Andreyev, A. Y., Kushnareva, Y. E., Murphy, A. N, Lonze, B. E, Kim, K. S., Ginty, D. D., Ferrante, R. J., Ryu, H. and Ratan, R. R. (2005) Mitochondrial cyclic AMP response element-binding protein (CREB) mediates mitochondrial gene expression and neuronal survival. J. Biol. Chem. 280: 40398-40401.

Lehman, J. J., Barger, P. M., Kovacs, A., Saffitz, J. E., Medeiros, D. M. and Kelly, D. P. (2000) Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. J. Clin. Invest. 106: 847-856.

Leick, L., Hellsten, Y., Fentz, J., Lyngby, S. S., Wojtaszewski, J. F., Hidalgo, J. and Pilegaard, H. (2009) PGC-1alpha mediates exercise-induced skeletal muscle VEGF expression in mice. Am. J. Physiology. 297: 92-103.

Li, L., Pan, R., Li, R., Niemann, B., Aurich, A. C., Chen, Y. and Rohrbach, S. (2011) Mitochondrial biogenesis and peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) deacetylation by physical activity: intact adipocytokine signaling is required. Diabetes 60: 157-167.

Lin, J., Wu, H., Tarr, P. T., Zhang, C. Y., Wu, Z., Boss, O., Michael, L. F, Puigserver, P., Isotani, E., Olson, E. N., Lowell, B. B, Bassel-Duby, R. and Spiegelman, B. M. (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 418: 797-801.

Liu, L., Zhang, W., Wang, L., Li, Y., Tan, B., Lu, X., Deng, Y., Zhang, Y., Guo, X., Mu, J. and Yu, G. (2014) Curcumin prevents cerebral ischemia reperfusion injury via increase of mitochondrial biogenesis. Neurochem. Res. 39: 1322-1331.

Ljubicic, V., Joseph, A. M., Saleem, A., Uguccioni, G., Collu, Marchese. M. and Lai, R. Y. J. (2010). Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: Effects of exercise and aging. Biochimica et Biophysica Acta (BBA) - General Subjects. 1800: 223-34.

Manach, C., Scalbert, A., Morand, C., Remesy, C. and Jimenez, L. (2004) Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79: 727-747.

Medeiros, D. M. (2008) Assessing mitochondria biogenesis. Methods 46: 288-294.

Meirhaeghe, A., Crowley, V., Lenaghan, C., Lelliott, C., Green, K., Stewart, A., Hart, K., Schinner, S., Sethi, J. K., Yeo, G., Brand, M. D., Cortright, R. N., O'Rahilly, S., Montague, C. and Vidal-Puig, A. J. (2003) Characterization of the human, mouse and rat PGC1 beta (peroxisome-proliferator-activated receptor-gamma co-activator 1 beta) gene in vitro and in vivo. Biochem. J. 373: 155-165.

Menzies, K. J., Singh, K., Saleem, A. and Hood, D. A. (2013) Sirtuin 1-mediated effects of exercise and resveratrol on mitochondrial biogenesis. J. Biol. Chem. 288: 6968-6979.

Middleton, E., Jr., Kandaswami, C. and Theoharides, T. C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52: 673-751.

Na, L. X., Zhang, Y. L., Li, Y., Liu, L. Y., Li, R., Kong, T. and Sun, C. H. (2011) Curcumin improves insulin resistance in skeletal muscle of rats. Nutr. Metab. Cardiovasc. Dis. 21: 526-533.

Nam, S. M., Choi, J. H., Yoo, D. Y., Kim, W., Jung, H. Y., Kim, J. W., Yoo, M., Lee, S., Kim, C. J., Yoon, Y. S. and Hwang, I. K. (2014) Effects of curcumin (Curcuma longa) on learning and spatial memory as well as cell proliferation and neuroblast differentiation in adult and aged mice by upregulating brain-derived neurotrophic factor and CREB signaling. J. Med. Food. 17: 641-649.

Narkar, V. A., Downes, M., Yu, R. T., Embler, E., Wang, Y. X., Banayo, E., Mihaylova, M. M., Nelson, M. C., Zou, Y., Juguilon, H., Kang, H., Shaw, R. J. and Evans, R. M. (2008) AMPK and PPARdelta agonists are exercise mimetics. Cell 134: 405-415.

Nemoto, S., Fergusson, M. M. and Finkel, T. (2005) SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1alpha. J. Biol. Chem. 280: 16456-16460.

Nisoli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello, C., Sciorati, C., Bracale, R., Valerio, A., Francolini, M., Moncada, S. and Carruba, M. O. (2003) Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science 299: 896-899.

Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., Falcone, S., Valerio, A., Cantoni, O., Clementi, E., Moncada, S. and Carruba, M. O. (2005) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310: 314-317.

Ohyama, K., Nogusa, Y., Suzuki, K., Shinoda, K., Kajimura, S. and Bannai, M. (2015) A combination of exercise and capsinoid supplementation

additively suppresses diet-induced obesity by increasing energy expenditure in mice. Am. J. Physiol. Endocrinol. Metab. 308: 315-23.

O'Neill, H. M., Holloway, G. P. and Steinberg, G. R. (2013) AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: implications for obesity. Mol. Cell. Endocrinol. 366: 135-151.

O'Neill, H. M., Maarbjerg, S. J., Crane, J. D., Jeppesen, J., Jorgensen, S. B., Schertzer, J. D., Shyroka, O., Kiens, B., Van-Denderen., B. J, Tarnopolsky., M. A., Kemp, B. E., Richter, E. A. and Steinberg, G. R. (2011) AMP-activated protein kinase (AMPK) beta1beta2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. Proc. Natl. Acad. Sci. 108: 16092-16097.

Palmer, W. K. (1988) Effect of exercise on cardiac cyclic AMP. Med. Sci. Sports. Exerc. 20: 525-530.

Park, S. J., Ahmad, F., Philp, A., Baar, K., Williams, T., Luo, H., Ke, H., Rehmann, H., Taussig, R., Brown, A. L., Kim, M. K., Beaven, M. A., Burgin, A. B., Manganiello, V. and Chung, J. H. (2012) Resveratrol Ameliorates Aging-Related Metabolic Phenotypes by Inhibiting cAMP Phosphodiesterases. Cell 148: 421-433.

Patti, M, E., Butte, A. J., Crunkhorn, S., Cusi, K., Berria, R., Kashyap, S., Miyazaki, Y., Kohane, I., Costello, M., Saccone, R., Landaker, E. J., Goldfine, A. B., Mun, E., DeFronzo, R., Finlayson, J., Kahn, C. R. and Mandarino, L. J. (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc. Natl. Acad. Sci. U. S. A. 100: 8466-8471.

Payton, F., Sandusky, P. and Alworth, W. L. (2007) NMR Study of the Solution Structure of Curcumin. J. Nat. Prod. 70: 143-146.

Petersen, K. F., Dufour, S., Befroy, D., Garcia, R. and Shulman, G. I. (2004) Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N. Engl. J. Med. 350: 664-671.

Philp, A., Chen, A., Lan, D., Meyer, G. A., Murphy, A. N., Knapp, A. E., Olfert, IM, McCurdy, C. E, Marcotte, G. R., Hogan, M. C., Baar, K. and Schenk, S. (2011) Sirtuin 1 (SIRT1) deacetylase activity is not required for mitochondrial biogenesis or peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) deacetylation following endurance exercise. J. Biol. Chem. 286: 30561-30570.

Price, N. L., Gomes, A. P., Ling, A. J. Y., Duarte, F. V, Martin, M. A., North, B. J., Agarwal, B., Ye, L., Ramadori, G., Teodoro, J. S., Hubbard, B. P., Varela, A. T., Davis, J. G., Varamini, B., Hafner, A., Moaddel, R., Rolo, A. P., Coppari, R., Palmeira, C. M., de Cabo, R., Baur, J. A. and Sinclair,

D. A. (2012) SIRT1 Is Required for AMPK Activation and the Beneficial Effects of Resveratrol on Mitochondrial Function. Cell. Metabolism. 15: 675-690.

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M. and Spiegelman, B. M. (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 92: 829-839.

Queen, B. L. and Tollefsbol, T. O. (2010) Polyphenols and aging. Curr. Aging. Sci. 3: 34-42

Revollo, J. R., Korner, A., Mills, K. F., Satoh, A., Wang, T., Garten, A., Dasgupta, B., Sasaki, Y., Wolberger, C., Townsend, R. R., Milbrandt, J., Kiess, W. and Imai, S. (2007) Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. Cell Metab. 6: 363-375.

Rodgers, J. T., Lerin, C., Gerhart, H, Z., Puigserver, P. (2008) Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. FEBS. Lett. 582: 46-53.

Rodgers, J. T., Lerin, C., Haas, W., Gygi, S. P., Spiegelman, B. M. and Puigserver, P. (2005) Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 434: 113-118.

Rouse, M., Younes, A. and Egan, J. M. (2014) Resveratrol and curcumin enhance pancreatic beta-cell function by inhibiting phosphodiesterase activity. J. Endocrinol. 223: 107-117.

Roy, R. R., Hutchison, D. L., Pierotti, D. J., Hodgson, J. A., Edgerton, V. R. (1985) EMG patterns of rat ankle extensors and flexors during treadmill locomotion and swimming. J. Appl. Physiol. 70: 2522-2529.

Russell, A. P., Feilchenfeldt, J., Schreiber, S., Praz, M., Crettenand, A., Gobelet, C., Meier, C. A., Bell, D. R., Kralli, A., Giacobino, J. P. and Deriaz, O. (2003) Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. Diabetes 52: 2874-2881.

Russell, L. K., Mansfield, C. M., Lehman, J. J., Kovacs, A., Courtois, M., Saffitz, J. E., Medeiros, D. M., Valencik, M. L., McDonald, J. A. and Kelly, D. P. (2004) Cardiac-specific induction of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator-1alpha promotes mitochondrial biogenesis and reversible cardiomyopathy in a developmental stage-dependent manner. Circ. Res. 94: 525-533.

Ryu, H., Lee, J., Impey, S., Ratan, R. R. and Ferrante, R. J. (2005) Antioxidants modulate mitochondrial PKA and increase CREB binding to D-loop DNA of the mitochondrial genome in neurons. Proc. Natl. Acad. Sci. 102: 13915-13920.

Sack, M. N. and Finkel, T. (2012) Mitochondrial metabolism, sirtuins, and aging. Cold Spring Harb. Perspect. Biol. 4: a013102

Sakamoto, K., McCarthy, A., Smith, D., Green, K. A., Grahame, H. D., Ashworth, A. and Alessi, D. R. (2005) Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. Embo. J. 24: 1810-1820.

Santos, A. C., Uyemura, S. A., Lopes, J. L., Bazon, J. N., Mingatto, F. E. and Curti, C. (1998) Effect of naturally occurring flavonoids on lipid peroxidation and membrane permeability transition in mitochondria. Free. Radic. Biol. Med. 24: 1455-1461.

Scapagnini, G., Foresti, R., Calabrese, V., Giuffrida, S. A. M., Green, C. J. and Motterlini, R. (2002) Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. Mol. Pharmacol. 61: 554-561.

Scarpulla, R. C. (2011) Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. Biochimica et Biophysica Acta. (BBA) - Mol. Cell. Res. 1813: 1269-1278.

Sheng, B., Wang, X., Su, B., Lee, H. G., Casadesus, G., Perry, G. and Zhu, X. (2012) Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in Alzheimer's disease. J. Neurochem. 120: 419-429.

Sherratt, H. S. (1991) Mitochondria: structure and function. Rev. Neurol. 147: 417-430.

Shetty, D., Kim, Y., Shim, H. and Snyder, J. (2014) Eliminating the Heart from the Curcumin Molecule: Monocarbonyl Curcumin Mimics (MACs). Molecules 20: 249.

Soriano, F. X., Liesa, M., Bach, D., Chan, D. C., Palacin, M. and Zorzano, A. (2006) Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha, estrogen-related receptor-alpha, and mitofusin 2. Diabetes 55: 1783-1791.

Srere, P. A. (1969) [1] Citrate synthase: [EC 4.1.3.7. Citrate oxaloacetate-lyase (CoA-acetylating)]. In Methods in Enzymology, John ML (ed) 13: 3-11. Academic Press.

St-Pierre, J., Lin, J., Krauss, S., Tarr, P. T., Yang, R., Newgard, C. B. and Spiegelman, B. M. (2003) Bioenergetic analysis of peroxisome

proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. J. Biol. Chem. 278: 26597-26603.

Stapleton, D., Woollatt, E., Mitchelhill, K. I., Nicholl, J. K., Fernandez, C. S., Michell, B. J., Witters, L. A., Power, D. A., Sutherland, G. R. and Kemp, B. E. (1997) AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location. FEBS. Letters 409: 452-456.

Stein, S. C., Woods, A., Jones, N. A., Davison, M. D. and Carling, D. (2000) The regulation of AMP-activated protein kinase by phosphorylation. Biochem. J. 3: 437-443.

Suwa, M., Nakano, H., Radak, Z. and Kumagai, S. (2008) Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor γ coactivator-1α protein expressions in rat skeletal muscle. Metabolism 57: 986-998.

Tanner, C. B., Madsen, S. R., Hallowell, D. M., Goring, D. M., Moore, T. M., Hardman, S. E., Heninger, M. R., Atwood, D. R. and Thomson, D. M. (2013) Mitochondrial and performance adaptations to exercise training in mice lacking skeletal muscle LKB1. Am. J. Physiol. Endocrinol. Metab.305: 27.

Taylor, E. B., Lamb, J. D., Hurst, R. W., Chesser, D. G., Ellingson, W. J., Greenwood, L. J., Porter, B. B., Herway, S. T. and Winder, W. W. (2005) Endurance training increases skeletal muscle LKB1 and PGC-1alpha protein abundance: effects of time and intensity. Am. J. Physiol. Endocrinol. Metab. 289: E960-968.

Terada, S., Goto, M., Kato, M., Kawanaka, K., Shimokawa, T. and Tabata, I. (2002) Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochem. Biophys. Res. Commun. 296: 350-354.

Than, T. A., Lou, H., Ji, C., Win, S. and Kaplowitz, N. (2011) Role of cAMP-responsive element-binding protein (CREB)-regulated transcription coactivator 3 (CRTC3) in the initiation of mitochondrial biogenesis and stress response in liver cells. J. Biol. Chem. 286: 22047-22054.

Uguccioni, G., Hood, D. A. (2011) The importance of PGC-1alpha in contractile activity-induced mitochondrial adaptations. Am J Physiology Endocrinol. Metab. 300: 361-371.

Um, J. H., Park, S. J., Kang, H., Yang, S., Foretz, M., McBurney, M. W., Kim, M. K., Viollet, B. and Chung, J. H. (2010) AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol. Diabetes 59: 554-563.

Veeranki, S., Hwang, S. H., Sun, T., Kim, B. and Kim, L. (2011) LKB1 regulates development and the stress response in Dictyostelium. Dev. Biol. 360: 351-357.

Ventura, C. R., Garnier, A. and Veksler, V. (2008) Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. Cardiovasc. Res. 79: 208-217.

Viña, J., Gomez, C. M. C., Borras, C., Froio, T., Sanchis, G. F., Martinez, B. V. E. and Pallardo, F. V. (2009) Mitochondrial biogenesis in exercise and in ageing. Adv. Drug. Deliv. Rev. 61: 1369-1374.

Wang, Y., Vera, L., Fischer, W. H. and Montminy, M. (2009) The CREB coactivator CRTC2 links hepatic ER stress and fasting gluconeogenesis. Nature 460: 534-537.

Wenz, T., Rossi, S. G., Rotundo, R. L., Spiegelman, B. M. and Moraes, C. T. (2009) Increased muscle PGC-1alpha expression protects from sarcopenia and metabolic disease during aging. Proc. Natl. Acad. Sci. 106: 20405-20410.

Wiesner, R. (1997) Adaptation of Mitochondrial Gene Expression to Changing Cellular Energy Demands. Physiol. 12: 178-184.

Williams, R. S., Salmons, S., Newsholme, E. A., Kaufman, R. E. and Mellor, J. (1986) Regulation of nuclear and mitochondrial gene expression by contractile activity in skeletal muscle. J. Biol. Chem. 261: 376-380.

Winder, W. W., Holmes, B. F., Rubink, D. S., Jensen, E. B., Chen, M. and Holloszy, J. O. (1985) Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. J. Appl. Physiol. 88: 2219-2226

Wu, H., Kanatous, S. B., Thurmond, F. A., Gallardo, T., Isotani, E., Bassel, D. and R., Williams, R. S. (2002) Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. Science 296: 349-352.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowel, B., Scarpulla, R. C. and Spiegelman, B. M. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98: 115-124.

Yang, H., Baur, J. A., Chen, A., Miller, C., Adams, J. K., Kisielewski, A., Howitz, K. T., Zipkin, R. E. and Sinclair, D. A. (2007) Design and synthesis of compounds that extend yeast replicative lifespan. Aging cell 6: 35-43.

Yang, Y., Duan, W., Lin, Y., Yi, W., Liang, Z., Yan, J., Wang, N., Deng, C., Zhang, S., Li, Y., Chen, W., Yu, S., Yi, D. and Jin, Z. (2013) SIRT1 activation by curcumin pretreatment attenuates mitochondrial oxidative damage induced by myocardial ischemia reperfusion injury. Free. Radic. Biol. Med. 65: 667-679.

Zong, H., Ren, J. M., Young, L. H., Pypaert, M., Mu, J., Birnbaum, M. J. and Shulman, G. I. (2002) AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. Proc. Natl. Acad. Sci. 99: 15983-15987.

Paper Reference

(1) Hamidie, R. D. R., Yamada, T., Ishizawa, R., Saito, Y. and Masuda, K. (2015) Curcumin treatment enhances the effect of exercise on mitochondrial biogenesis in skeletal muscle by increasing cAMP levels. Metabolism. 64: 1334-1347

Available online at www.sciencedirect.com

Metabolism

www.metabolismjournal.com

Translational

Curcumin treatment enhances the effect of exercise on mitochondrial biogenesis in skeletal muscle by increasing cAMP levels

Ronald D. Ray Hamidie[a,b], Tatsuya Yamada[a], Rie Ishizawa[a], Yoko Saito[c], Kazumi Masuda[a,d,*]

[a] Graduate School of Natural Science and Technology, Kanazawa University, Ishikawa 920-1192, Japan
[b] Faculty of Sport and Health Education, Indonesia University of Education, West Java 40154, Indonesia
[c] Faculty of Human Sciences, Kanazawa Seiryo University, Ishikawa 920-8620, Japan
[d] Faculty of Human Sciences, Kanazawa University, Ishikawa 920-1192, Japan

ARTICLE INFO

Article history:
Received 16 December 2014
Accepted 15 July 2015

Keywords:
Endurance training
Mitochondria
Polyphenol
Skeletal muscle
cAMP

ABSTRACT

Background. In response to physiologic stressors, skeletal muscle has the potential to elicit wide variety of adaptive responses, such as biogenesis of mitochondria and clearance of damaged mitochondria to promote healthy muscle. The polyphenol curcumin, derived from the rhizome *Curcuma longa L.*, is a natural antioxidant that exhibits various pharmacological activities and therapeutic properties. However, the effect of curcumin on the regulation of mitochondrial biogenesis in skeletal muscle remains unknown. The present study aimed to examine the effects of combination of endurance training (eTR) and curcumin treatment on the expression of AMPK, SIRT1, PGC-1α, and OXPHOS subunits, mitochondrial DNA copy number, and CS activity in rat skeletal muscle. Furthermore, the present study also examined the effect of exercise and curcumin treatment on the levels of cAMP and downstream targets of PKA including phosphorylated CREB and LKB-1.

Methods. Ten-week-old male Wistar rats were randomly divided into non-eTR and eTR groups. Low doses (50 mg/kg-BW/day) or high doses (100 mg/kg-BW/day) of curcumin dissolved in dimethyl sulfoxide (DMSO) were injected intraperitoneally in all animals for 28 days to investigate the effect of curcumin alone and the combined effect of curcumin with eTR. Western blotting (WB) and immunoprecipitation (IP) were performed to detect the presence of proteins.

Results. Our results demonstrated that combination of curcumin treatment and eTR increased the expression of COX-IV, OXPHOS subunits, mitochondrial DNA copy number and CS activity in the gastrocnemius (Gas) and soleus (Sol) muscles. In addition, this combination increased AMPK phosphorylation, NAD^+/NADH ratio, SIRT1 expression, and

Abbreviations: AMPK, 5′ adenosine monophosphate-activated protein kinase; BW, body weight; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; COX-IV, cytochrome c oxidase subunit IV; CS, citrate synthase; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; eTR, endurance training; Gas, gastrocnemius muscle; IP, immunoprecipitation; LKB-1, liver kinase B1; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrogen; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1alpha; PKA, protein kinase A; SIRT1, sirtuin 1; Sol, soleus muscle; WB, western blotting; GLUT4, Glucose transporter 4; NRF1/2, nuclear respiratory factor1/2; GA, guanine adenine; Tfam, transcription factor A; mRNA, messenger ribonucleid acid; ADP, adenosine diphosphate; MMP, mitochondrial membrane potential; ATP, adenosine triphosphate; ROS, reactive oxygen species; NOS, nitric oxide synthase.

* *Corresponding author at:* Faculty of Human Sciences, Kanazawa University, Kakuma-machi, Kanazawa-city, Ishikawa, 920-1192, Japan. Tel.: +81 76 264 5568; fax: +81 76 234 4100.
E-mail address: masudak@staff.kanazawa-u.ac.jp (K. Masuda).

http://dx.doi.org/10.1016/j.metabol.2015.07.010

PGC-1α deacetylation. Furthermore, curcumin treatment as well as exercise also increased levels of cAMP and downstream target of PKA including phosphorylation CREB and LKB-1 which are involved in the regulation of mitochondrial biogenesis.

Conclusion. Taken together, these results suggest that the combination of curcumin treatment and eTR has the potential to accelerate mitochondrial biogenesis in skeletal muscle by increasing cAMP levels.

1. Introduction

Skeletal muscle represents one of the largest organs in the body, with a wide variety of adaptive responses to physiological stressors. The biogenesis of mitochondria and the clearance of damaged mitochondria promote healthy muscle and this turnover can prevent metabolic imbalances, which could predispose individuals to the development of obesity, diabetes, cardiovascular disease, and accelerated aging [1,2]. Muscle adaptation occurs in the mitochondria following exercise training. Endurance exercise training has the potential to enhance metabolic characteristics in the skeletal muscle, including mitochondrial biogenesis and the expression of glucose transporter 4 (GLUT4) [3]. Although such adaptations have been investigated for several decades, their underlying mechanisms remain to be elucidated. Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) has been implicated as a master regulator of mitochondrial biogenesis by interacting with nuclear respiratory factor 1 (NRF1) and nuclear respiratory factor 2 (NRF-2/GA-binding protein-A). Both PGC-1α and NRF-1 activate the mitochondrial transcription factor A (Tfam), which is responsible for transcribing nuclear-encoded mitochondrial genes, as well as the transcription, translation, and repair of proteins involved in mitochondrial DNA (mtDNA) [4,5]. The expression levels of PGC-1α, (both mRNA and protein) increase following acute endurance exercise [6–9] and endurance exercise training [10,11], thus suggesting that PGC-1α is a potential regulator of metabolic adaptations after endurance exercise.

Mammalian sirtuins, such as sirtuin 1 (SIRT1), are members of a conserved family of NAD^+-dependent deacetylases and ADP-ribosyltransferases, which are involved in numerous fundamental cellular processes, including gene silencing, DNA repair, and metabolic regulation [12,13]. SIRT1 promotes mitochondrial biogenesis via the deacetylation of PGC-1α. Another important metabolic sensor, AMP-activated protein kinase (AMPK), also activates PGC-1α. Both SIRT1 expression [14] and activity [15] increase after endurance exercise. SIRT1 is also activated by acute endurance exercise [16] and it has been proposed that AMPK activates SIRT1 indirectly by increasing the intracellular levels of its co-substrate, NAD^+. Similar to AMPK, SIRT1 is widely associated with energy metabolism, suggesting that activators of these enzymes could enhance mitochondrial biogenesis and function and thereby enhance the endurance capacity. Collectively, these findings suggest that metabolic adaptations resulting from endurance exercise training could result, at least in part, from an AMPK-mediated increase in the expression of PGC-1α.

In both mammalian cells and yeast, the regulation of mitochondrial biogenesis clearly involves the cyclic adenosine monophosphate (cAMP) signaling pathway. Indeed, it has been shown that treatment of human preadipocytes with forskolin, which leads to an over-activation of the cAMP pathway, increased the copy number of mtDNA [17]. Protein kinase A (PKA), also known as cAMP-dependent enzyme, is a well studied, downstream effector of cAMP and is activated only in the presence of cAMP. Activated PKA phosphorylates a number of other proteins including cAMP response element binding protein (CREB) and liver kinase B1 (LKB-1), which induces PGC-1α to regulate mitochondria biogenesis ([18,19]. Previous studies have provided evidence that exercise, including swimming and running increase cAMP levels in skeletal muscle and myocardium [20] and the mitochondrial biogenesis seen after exercise may be attributable to this increase.

Several polyphenols have been shown to activate cAMP, and are currently under intense investigation as potential inducers of mitochondrial biogenesis [21,22]. Curcumin, a compound with medicinal properties found in *Curcuma longa L*, and turmeric, a popular culinary spice used in both vegetarian and non-vegetarian foods are examples of polyphenols. The anti-oxidant activities of curcumin have been reported to be more potent than those of another well-studied polyphenol, resveratrol [23] and the long-term effects of dietary curcumin on various markers of mitochondrial biogenesis have been investigated. Five months of curcumin as a dietary supplement in senescence-accelerated mouse-prone 8 (SAMP8), a fast-aging mouse strain, up-regulated PGC-1α protein expression thereby improving the mitochondrial membrane potential (MMP) and ATP levels and restoring mitochondrial fusion in the brain [24]. The effects of curcumin on the regulation of mitochondrial biogenesis in skeletal muscle however, have yet to be elucidated.

In the present study, we investigated the mechanisms by which curcumin affects mitochondrial biogenesis in rats as reflected by the levels of cAMP and downstream targets of PKA including phosphorylated CREB and LKB-1. The primary purpose of the present study was to determine the combined effects of curcumin treatment together with 24 days of endurance training (eTR) on the regulation of mitochondrial biogenesis in skeletal muscles (Gas and Sol). We predicted that treatment with curcumin combined with eTR may additively or synergistically enhance the effect of exercise. The results showed that curcumin treatment and eTR have additive effect on increasing mitochondrial biogenesis in skeletal muscle.

2. Material and Methods

2.1. *Animals Experiments*

All procedures performed in the present study were approved by the Ethics Committee on Animal Experimentation of

Kanazawa University (Protocol: AP-10187). Thirty six ten-weeks-old male Wistar rats (body weight, 282–390 g) were used (six rats in every group). The animals were housed in an air-conditioned room and exposed to a 12-h light-dark photoperiod. A standard diet (Oriental Yeast, Tokyo, Japan) and water were provided *ad libitum*. The animals were randomly divided into six groups: control without eTR, curcumin 50 mg/kg-BW/day without eTR, curcumin 100 mg/kg-BW/day without eTR, control with eTR, curcumin 50 mg/kg-BW/day with eTR, and curcumin 100 mg/kg-BW/day with eTR. All animals were injected intraperitoneally (I.P), once a day for twenty-eight days with curcumin (50 or 100 mg/kg-BW/day) dissolved in dimethyl sulfoxide (DMSO) or the same volume of DMSO (vehicle alone). The endurance exercise training (eTR) group rats swam 2 h/day in four 30-min bouts separated by 5 min of rest. After the first 30-min bout, a weight equal to 2% of body weight was tied to the body of the rat. The rats swam with the weight attached for the remaining three exercise bouts. All rats swam in a barrel filled to a depth of 50 cm with a swimming area of 190 cm^2/rat [25]. The rats performed the above swimming protocol once a day for 24 days (6 days/week × 4 weeks). For studies involving the effect of curcumin on the levels of cAMP and PKA, ten-weeks-old male Wistar rats (n = 6 in each group) and the same swimming protocol were used for 3 days of treatment and H89 (a PKA inhibitor) was injected I.P (20 mg/kg-BW/day) for 3 days.

2.2. Nuclear Fraction Preparation

Animals were anesthetized with 50 mg of pentobarbital sodium per 100 g of body weight, one hour after the last endurance exercise session. For biochemical studies, two calf muscles (Gas and Sol) were isolated; the tissues were washed in ice-cold saline, separated from the connective tissues and nerves, and then frozen rapidly in liquid nitrogen. Nuclear proteins were isolated using a modified version of the protocol established by Blough [26] and divided into 1-ml PBS aliquots.

2.3. Immunoprecipitation and Western Blotting

Lysis buffer (20 mM Tris-HCl [pH 7.4], 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM PMSF) was used for immunoprecipitation. Dynabeads (Invitrogen, CA, USA) were added to the samples of nuclear proteins, and incubated at 4 °C for 1 h. The Dynabeads were then collected using magnets, and normal mouse immunoglobulin G (nIgG; Santa Cruz Biotechnology, CA, USA) was added to the supernatants and incubated at 4 °C overnight. Samples were centrifuged for 30 s × 7000×*g* at 4 °C, and aliquots of this supernatant were reacted with primary antibodies against PGC-1α and mouse nIgG at 4 °C overnight. Dynabeads were then added and incubated at 4 °C for 2 h. The pellets were collected using magnets and washed with PBS. The final pellet from the immunoprecipitation (IP) was visualized and the expression of proteins was compared by western blotting. Western blotting analysis was performed as previously described [27]. Briefly, equal protein amounts of samples were loaded onto SDS-PAGE gels 7.5% (SIRT1, PGC-1α, and acetylated lysine), 10% (phospho-AMPKα, AMPKα, phospho-CREB, CREB + CREM, phospho-LKB-1 and LKB-1), and 12.5% (COX-IV, Total OXPHOS), and the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated in blocking buffer, and subsequently with PGC-1α (1:500 dilution, #: KP9803-D00151286, Calbiochem, San Diego, CA, USA), acetylated–lysine (1:1000 dilution, #: 94415-11, Cell Signaling Technology, Danvers, MA, USA). Phospho-AMPKα (Thr 172) (1:1000 dilution, #: 2535S-16, Cell Signaling Technology, Danvers, MA, USA), AMPKα (1:1000 dilution, #: 26035-6, Cell Signaling Technology, Danvers, MA, USA), SIRT1 (1:1000 dilution, #: 13181-1-AP-00014457, Proteintech, Chicago, IL, USA), COX-IV (1:1000 dilution, #: ab14744-GR125134-4, Abcam, Cambridge, England), Total OXPHOS (1:1500 dilution, #: MS604-G2830, Abcam, Cambridge, England), Phospho LKB-1 (S 428) (1:500 dilution, #: ab63473-GR91843-2, Abcam, Cambridge, England), LKB-1 (1:500 dilution, #: ab58786-GR146310-3, Abcam, Cambridge, England), Phospho CREB (Ser 133) (1:500 dilution, #: ab173780-GR316230-2, Abcam, Cambridge, England), CREB + CREM (1:500 dilution, #: ab5803-GR156230-3, Abcam, Cambridge, England), anti-β-actin (1:1000 dilution, #: ab8226-GR110198-3, Abcam, Cambridge, England), GAPDH (1:1000 dilution, #: ab8245-GR168547-1, Abcam, Cambridge, England), and lamin (1:1000 dilution, #: SC-20681-D0142, Santa Cruz Biotechnology, CA, USA). The signal intensity was quantified using imaging software (Image J, version 1.46, NIH, Maryland, USA).

2.4. NAD^+/NADH Ratio

The intracellular NAD^+/NADH ratio was assessed using an NAD^+/NADH assay kit (Biovision, CA, USA) according to the manufacturer's instructions. The absorbance ($OD_{450\ nm}$) was read 1 h after addition of the NADH developer, and the concentration of NAD^+ was calculated by subtracting the NADH concentration from the total value of NAD.

2.5. Citrate Synthase Activity

Skeletal muscle (20 mg) was homogenized on ice in 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.0. Citrate synthase (CS) activity was determined spectrophotometrically according to the method described by Srere [28]. The homogenates were frozen and thawed three times to disrupt the mitochondria to expose the CS and the assay was performed in a total volume of 900 μl: 100 mM Tris buffer (pH 8.0), 1 mM 5,5-dithiobis (2-nitrobenzoate) (DTNB), 3 mM acetyl-CoA, 5 mM oxaloacetate (OAA), and 100 μl of muscle homogenate. The principle of the assay is to initiate the reaction of acetyl-CoA with OAA and link the release of free CoA-SH to a colorimetric reagent, DTNB. All measurements were performed in duplicate at 30 °C. CS activity was normalized to the total protein content and was expressed as micromoles per gram protein per minute.

2.6. Mitochondrial DNA Copy Number

Genomic DNA was extracted using a spin column. Briefly, muscle tissue was treated with proteinase K (20 μl, Qiagen Inc., Valencia, CA), followed by addition of SDS (200 μl) to lysed the cells. The homogenized solution was incubated at

56 °C and 100% ethanol (200 μl) was added to precipitate the DNA. The mixture was applied to a QIAamp spin column, and after two washes with buffer (500 μl), genomic DNA was eluted with elution buffer (200 μl). The final DNA concentration was determined by UV spectrophotometry at 260 and 280 nm. The mitochondrial (mt) copy number was estimated by quantitative real-time PCR (ABI 7300 Real Time PCR System; Foster City, CA) from the relative amounts of nuclear and mtDNA. (mt:nuclear DNA) which reflects the concentration of mitochondria per cell in the tissue. The mt.DNA forward primer used was (5′–3′) CCCTAAAACCCGCC ACATCT and the reverse primer was GAGCGATGGTGAGAGC TAAGGT. For nuclear DNA, the forward primer was CGAGTCGTCTTT CTCCTGATGAT and the reverse primer was TTCTGGATTCCAA TGCTTCGA. Quantification was performed in a total reaction volume of 20 μl containing: 2X SYBR Tli RNaseH Plus (10 μl), each primer (0.8 μl), 50X ROX Reference Dye II (0.4 μl) sample DNA (2 μl), and water (6 μl). Amplification and detection were performed in a real time PCR system (7300 real time PCR system, Applied Biosystems). The PCR was initiated with 95 °C denaturation followed by an enzyme activation step for 30 s followed by 40 cycles of 95 °C denaturation for 5 s, and annealing and elongation for 30 s at 60 °C. Samples were assayed in duplicate. Data analysis was based on measurement of the cycle threshold (CT), and the difference in CT values was used as a measure of relative abundance: CT (nDNA) – CT (mtDNA) or ΔCT, a quantitative measure of the mitochondrial genome. Results were expressed as the copy number of mtDNA per cell, $2 \times 2^{-\Delta CT}$, which is a unitless ratio.

2.7. cAMP Level

Levels of cAMP in cell extracts were measured with the cAMP Direct Immunoassay Kit from Abcam (ab65355), following the manufacturer's instructions. Briefly, add 50 μl of the acetylated Standard cAMP and test samples from to the Protein G coated 96-well plate. This step following by added 10 μl of the reconstituted cAMP antibody per well to the standard cAMP and sample wells. Incubate for 1 hour at room temperature with gentle agitation. Wash 5 times with 200 μl 1X Assay Buffer each wash. Completely empty the wells by tapping the plate on a new paper towel after each wash. After making sure there is no liquid left behind, add 100 μl of HRP developer and develop for 1 hour at room temperature with agitation. Finally, stop the reaction by adding 100 μl of 1M HCl (not provided) to each well (sample color should change from blue to yellow) and read the plate at $OD_{450\ nm}$ by spectrophotometry (Infinite 200 PRO, Tecan, USA).

2.8. Statistical Analysis

Two-way analysis of variance (ANOVA) as used to assess the main effect of exercise (non-eTR vs. eTR groups) and curcumin (non-curcumin vs. curcumin groups) and the exercise × curcumin interaction. The Tukey-Kramer post-hoc test was used for analysis to identify of difference between low dose and high dose curcumin groups. All data were expressed as mean ± standard deviation (SD). The level of significance was established at $P < 0.05$.

3. Results

3.1. Effect of Curcumin Treatment on Body Weight

Descriptive data for body weight are presented in Table 1. Two-way ANOVA indicated that eTR decreased body weight compared to non-eTR ($F = 45.91$, $P < 0.001$). Furthermore rats with curcumin treatment also decreased body weight compare to non-curcumin group ($F = 15.85$, $P < 0.001$) with no interaction between two factors ($F = 3.90$, $P > 0.05$). Post-hoc test analysis indicated that only high dose curcumin treatment was significantly different than DMSO ($P < 0.05$). These results indicated that curcumin and eTR have additive effect to reducing body weight.

3.2. Curcumin Treatment with eTR Enhances Expression of the Mitochondrial Markers COX-IV and OXPHOS Subunit

We assessed the mitochondrial content in skeletal muscle by measuring the expression level of cytochrome c oxidase subunit IV (COX-IV). Figs. 1A and 2A show the change in the COX IV protein expression in skeletal muscle after 28 days of treatment with eTR or curcumin treatment. Two-way ANOVA showed significantly different effect for eTR in Gas and Sol ($F = 173.03$ for Gas, 150.75 for Sol, $P < 0.001$) and curcumin treatment ($F = 36.94$ for Gas, 20.77 for Sol, $P < 0.001$) with no interaction between two factors (n.s.). Furthermore, post-hoc test analysis indicated that both doses of curcumin were significantly different than DMSO ($P < 0.05$) and high dose and low dose were significantly different ($P < 0.05$). The results of four representative oxidative phosphorylation (OXPHOS) subunits by two-way ANOVA analysis demonstrated that eTR upregulated the protein levels, in both Gas and Sol (Figs. 1B and C, 2B and C, $P < 0.001$) but was not showed for Complex V: ATP synthase subunit-α (Figs. 1D and 2D, n.s.). Furthermore, in both Gas and Sol, curcumin treatment increased Complex I subunit NDUFB8 ($F = 16.92$ for Gas, 131.71 for Sol, $P < 0.001$)

Table 1 – Description data for body weight.

Body weight			
GROUPS	Before exercise (g)	After Exercise 28 days (g)	Δ
DMSO	352.3 ± 9.6	399.2 ± 13.6	46.8 ± 5.8
CD 50	341.5 ± 32.3	380.8 ± 31.2	39.3 ± 32.8
CD 100	387.3 ± 14.2	420.2 ± 13.6	32.8 ± 5.2
DMSO + eTR	330 ± 35.6	359.3 ± 35.7	29.3 ± 4.5
CD 50 + eTR	331.2 ± 26,6	364.7 ± 26.1	33.5 ± 5.5
CD 100 + eTR	335.8 ± 18.7	359.3 ± 13.2	21.5 ± 6.1

Value are mean ± SD (n = 6 in each group), DMSO = control without endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. DMSO + eTR = control with endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO + endurance exercise. CD 100 = curcumin 100 mg/kg-BW/day in DMSO + endurance exercise. Two-way ANOVA revealed main effect for eTR ($P < 0.001$) and curcumin treatment ($P < 0.001$) with no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, only high doses curcumin was significantly decreased body weight ($P < 0.05$).

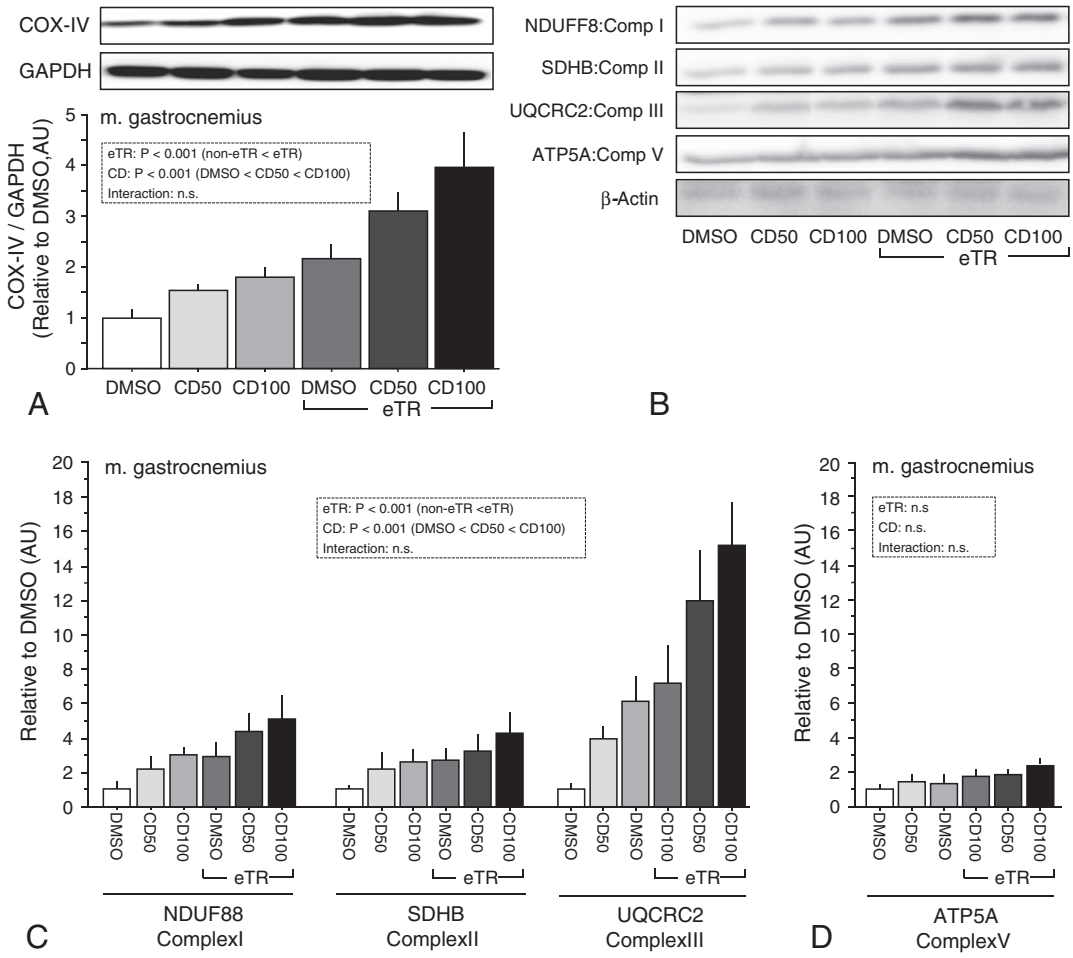


Fig. 1 – COX-IV protein expression (A), OXPHOS subunits expression (B) and intensity OXPHOS subunits relative to DMSO (C and D) in gastrocnemius.Value are mean ± SD (n = 6 in each group) DMSO = control without endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed the main effect for eTR (P < 0.001) and curcumin treatment (P < 0.001) for COX-IV and OXPHOS subunit expression in Gas with no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses of curcumin increased COX IV (A) and OXPHOS subunit Complex I, II, and III expression (B and C, P < 0.05) in Gas.

with no interaction between two factors (n.s.), Complex II subunit 30 kDa (F = 10.59 for Gas, 13.68 for Sol, P < 0.001) with no interaction between two factors (n.s.), and Complex III subunit Core 2 (F = 35.33 for Gas, 60.229 for Sol, P < 0.001) with no interaction between two factors (n.s.), whereas levels of Complex V: ATP synthase subunit-α was not increased in Gas and Sol (Figs. 1D and 2D, n.s.). Additionally, post-hoc analysis indicated that high dose curcumin was significantly different than low dose (P < 0.05) for increasing OXPHOS subunit Complex I, II, III and COX-IV in Gas and Sol (P < 0.05). In conclusion, these results indicated that curcumin treatment in conjunction with eTR increased the expression of COX-IV and OXPHOS subunits in skeletal muscle and curcumin and eTR were found to act additively.

3.3. *Curcumin Treatment Together with eTR Enhances the Mitochondrial DNA Copy Number and CS Activity*

An increase in mtDNA copy number relative to nuclear DNA is one of the best molecular markers of mitochondrial biogenesis [22]. Two way ANOVA demonstrated that eTR significantly increased the mtDNA copy number in both Gas and Sol compared to non eTR (Fig. 3A, F = 593.82 for Gas, 145.94 for Sol, P < 0.001), curcumin treatment increased mtDNA copy number compared to that by the control only DMSO (Fig. 3A, F = 43.46 for Gas, 14.05 for Sol, P < 0.001) with no interaction between two factors (n.s.). Furthermore, post-hoc test analysis indicated that both doses of curcumin were significantly different than DMSO group (P < 0.05) in Gas but in Sol only high dose significantly different than DMSO (P < 0.05). In addition, eTR increased CS enzyme activity in Gas and Sol (Fig. 3B, F = 652.69 for Gas, 152.09 for Sol, P < 0.001) and this was observed also with curcumin treatment where the CS activity increased in both Gas and Sol (Fig. 3B, F = 42.34 for Gas, 38.04 for Sol, P < 0.001) with no interaction between two factors (n.s.). Post-hoc analysis indicated that only high dose curcumin was significantly different than DMSO (P < 0.05) to enhance CS activity in Gas and Sol (P < 0.05). These results indicate that high-dose curcumin treatment and eTR additively increases the mitochondrial copy number and CS activity in skeletal muscle.

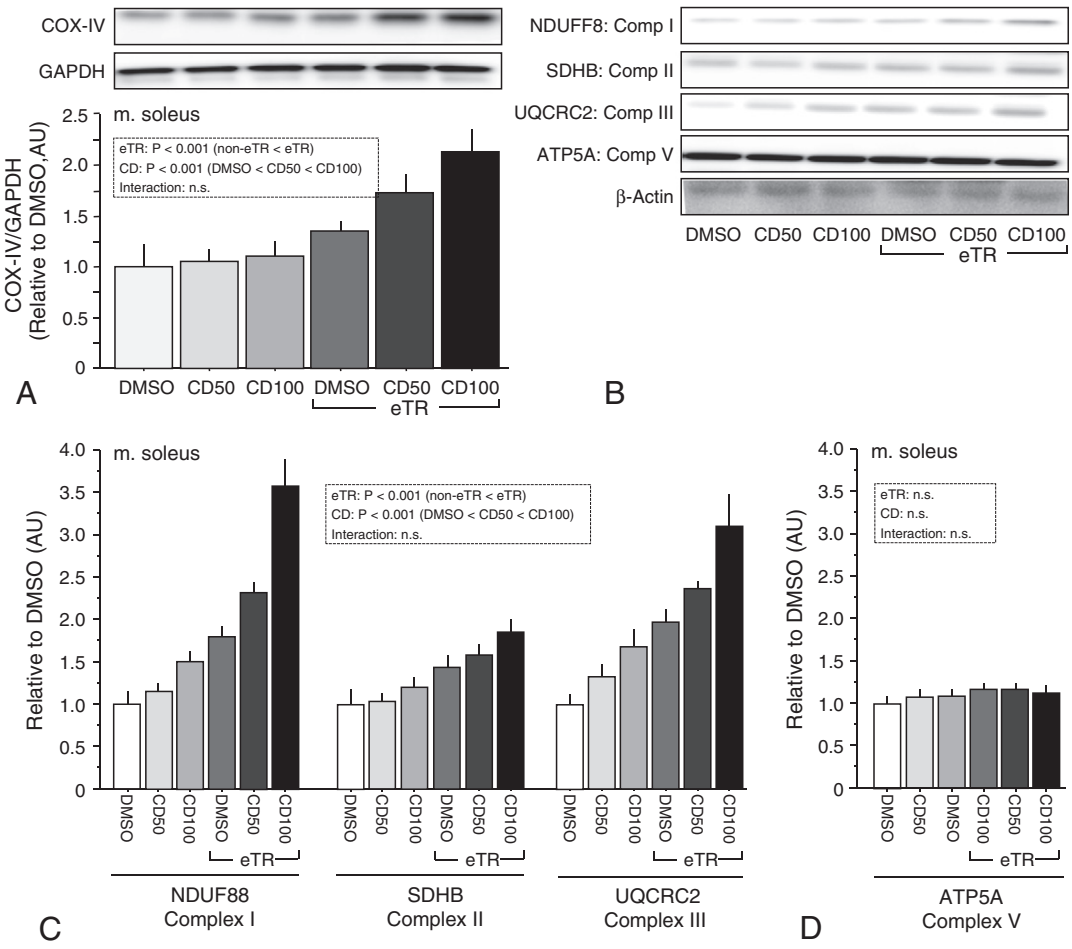


Fig. 2 – COX-IV protein expression (A), OXPHOS subunits expression (B) and intensity OXPHOS subunits relative to DMSO (C and D) in soleus.Value are mean ± SD (n = 6 in each group) DMSO = control without endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. Two-way ANOVA revealed the main effect for eTR (P < 0.001) and curcumin treatment (P < 0.001) for COX-IV (A) and OXPHOS subunit (B and C) expression in Sol with no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses of curcumin increased COX IV (A) and OXPHOS subunit Complex I, II, and III expression (B and C, P < 0.05) in Sol.

3.4. *Curcumin Treatment with eTR Increases Phosphorylation AMPK*

AMPK is a critical regulator of mitochondrial biogenesis in response to energy deprivation and previous studies have reported that its expression increases after exercise [29,30]. Furthermore, previous studies found that curcumin increases the phosphorylation of AMPK in skeletal muscle [31]. In accordance with previous study reports, two way ANOVA demonstrated that administration of curcumin enhanced the phosphorylation of the Thr^{172} residue of the AMPKα catalytic subunit in Gas and Sol (Fig. 4A and B, F = 100.51 for Gas, 17.99 for Sol, P < 0.001). In both Gas and Sol, eTR treatment increased phosphorylation of AMPK (Fig. 4A and B, F = 383.29 for Gas, 125.96 for Sol, P < 0.001) with no interactions between two factors (n.s.). Meanwhile, post-hoc analysis showed that both doses curcumin were significantly different than DMSO (Fig. 4A and B, P < 0.05) and low dose was different than high dose (P < 0.05) to increase phosphorylation AMPK in Gas and Sol (Fig. 4A and B, P < 0.05). This result suggests that curcumin activates AMPK phosphorylation in skeletal muscles and that the effect is in addition to the response to eTR.

3.5. *Curcumin Treatment with eTR Increases NAD^{+}/NADH Ratio and SIRT1 Expression*

NAD^{+} and NADH, are coenzymes required for the generation of ATP by mitochondria. SIRT1 is dependent on NAD^{+} as a substrate and has therefore been proposed as a key regulator of the adaptation to acute and chronic exercise in the mitochondria of skeletal muscle [32]. In accordance with this approach, two way ANOVA demonstrated that eTR increased the cellular NAD^{+}/NADH ratio in Gas and Sol (Fig. 5A and B, F = 243.73 for Gas, 587.07 for Sol, P < 0.001). Furthermore, curcumin treatment increased the cellular NAD^{+}/NADH ratio in Gas and Sol (Fig. 5A and B, F = 39.74 for Gas, 50.97 for Sol, P < 0.001) with no interaction between two factors (n.s.). However, curcumin treatment increased the expression of the SIRT1 protein only in Gas (Fig. 5C, F = 14.32, P < 0.001) but not Sol (Fig. 5D, n.s.) and eTR increased SIRT1 protein

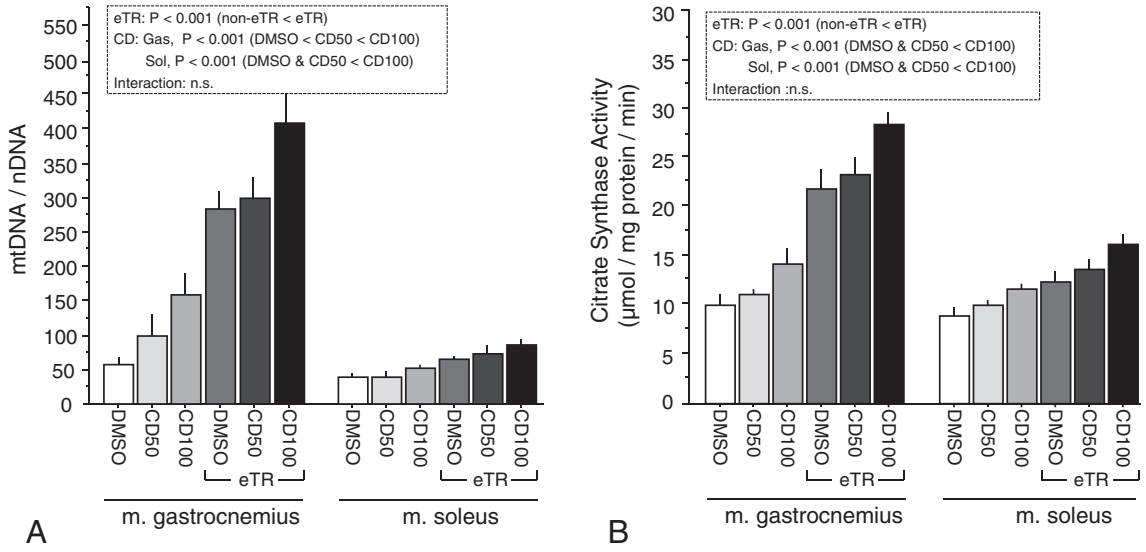


Fig. 3 – Mitochondria DNA copy number (A) and CS activity (B) in skeletal muscle.Value are mean ± SD (n = 6 in each group) DMSO = control without endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. eTR = endurance training. (A) Two-way ANOVA revealed the main effect for eTR (P < 0.001) and curcumin treatment (P < 0.001) for mitochondrial DNA copy number in Gas and Sol with no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses of curcumin increased mitochondrial copy number in Gas (P < 0.05) but in Sol only for high dose curcumin. (B) Two-way ANOVA revealed main effect for eTR (P < 0.001) and curcumin treatment (P < 0.001) for CS activity in Gas and Sol and no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, high dose of curcumin increased CS activity in Gas and Sol (P < 0.05).

expression in both muscle types (Gas and Sol) (Fig. 5C and D, F = 75.97 for Gas, 53.65 for Sol, P < 0.001) with no interaction between the two factors (n.s.). Meanwhile, post-hoc analysis showed that both doses of curcumin were significantly different than DMSO to increase NAD^+/NADH ratio in both skeletal muscles (Fig. 5A and B, P < 0.05). On the other hand, only high dose curcumin was different than DMSO to increase SIRT1 protein expression in Gas (Fig. 5C, P < 0.05). These results

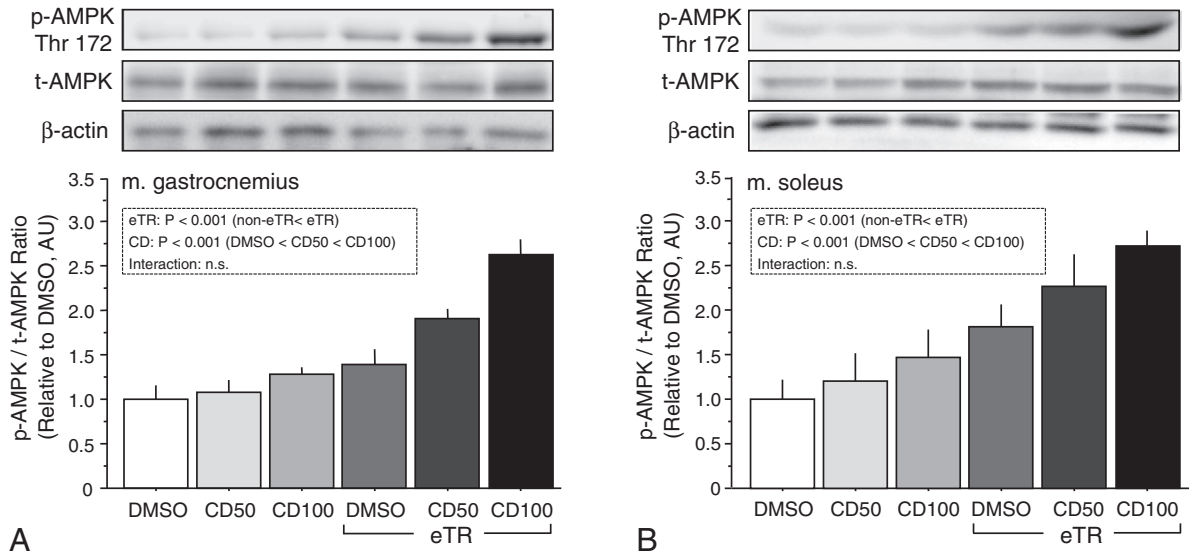


Fig. 4 – AMPK phosphorylation in skeletal muscle.Value are mean ± SD (n = 6 in each group) DMSO = control without endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. DMSO + eTR = control with endurance exercise. eTR = endurance training. Two-way ANOVA revealed the main effect for eTR (P < 0.001) and curcumin treatment (P < 0.001) for phosphorylation of AMPK in Gas (A) and Sol (B) with no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses of curcumin increased phosphorylation of AMPK in skeletal muscle (P < 0.05).

indicate that high dose curcumin increases the expression of SIRT1 in skeletal muscle via an increase in the cellular NAD^+/NADH ratio and that this is additive to the effect of eTR.

3.6. *Curcumin Treatment with eTR Enhances Deacetylation of PGC-1α*

Activation of PGC-1α is important both for the determination of mitochondrial content and the induction of mitochondrial biogenesis in skeletal muscle. Previous research has demonstrated that deacetylation of PGC-1α by SIRT1 is important for skeletal muscle function and the induction of mitochondrial biogenesis in response to exercise [32]. We therefore examined the deacetylation of PGC-1α in skeletal muscle. As shown in Fig. 6A and B, two way ANOVA determined that curcumin treatment, decreased the acetylation of PGC-1α in Gas and Sol (Fig. 6A and B, F = 26.03 for Gas, 14.09 for Sol, P < 0.001). In addition, eTR decreased the acetylation of PGC-1α in both Gas and Sol (Fig. 6A and B, F = 148.82 for Gas, 217.923 for Sol, P < 0.001) with no interaction between the two factors (n.s.). Furthermore, post-hoc analysis showed that low dose and high dose curcumin were significantly different than DMSO in Gas and Sol (Fig. 6A and B, P < 0.05) but there was no different between low dose and high dose treatment (n.s.). These results together indicate that curcumin induced PGC-1α deacetylation in skeletal muscles in an additive manner to the effect produced by eTR alone.

3.7. *Curcumin Treatment Increases cAMP Level*

It is well known that exercise increases the levels of cAMP [33] and curcumin has also been reported to have this effect as measured by inhibition of endothelial cell phosphodiesterase (PDE) [34]. Curcumin indeed increased cAMP levels (Fig. 7A, F =

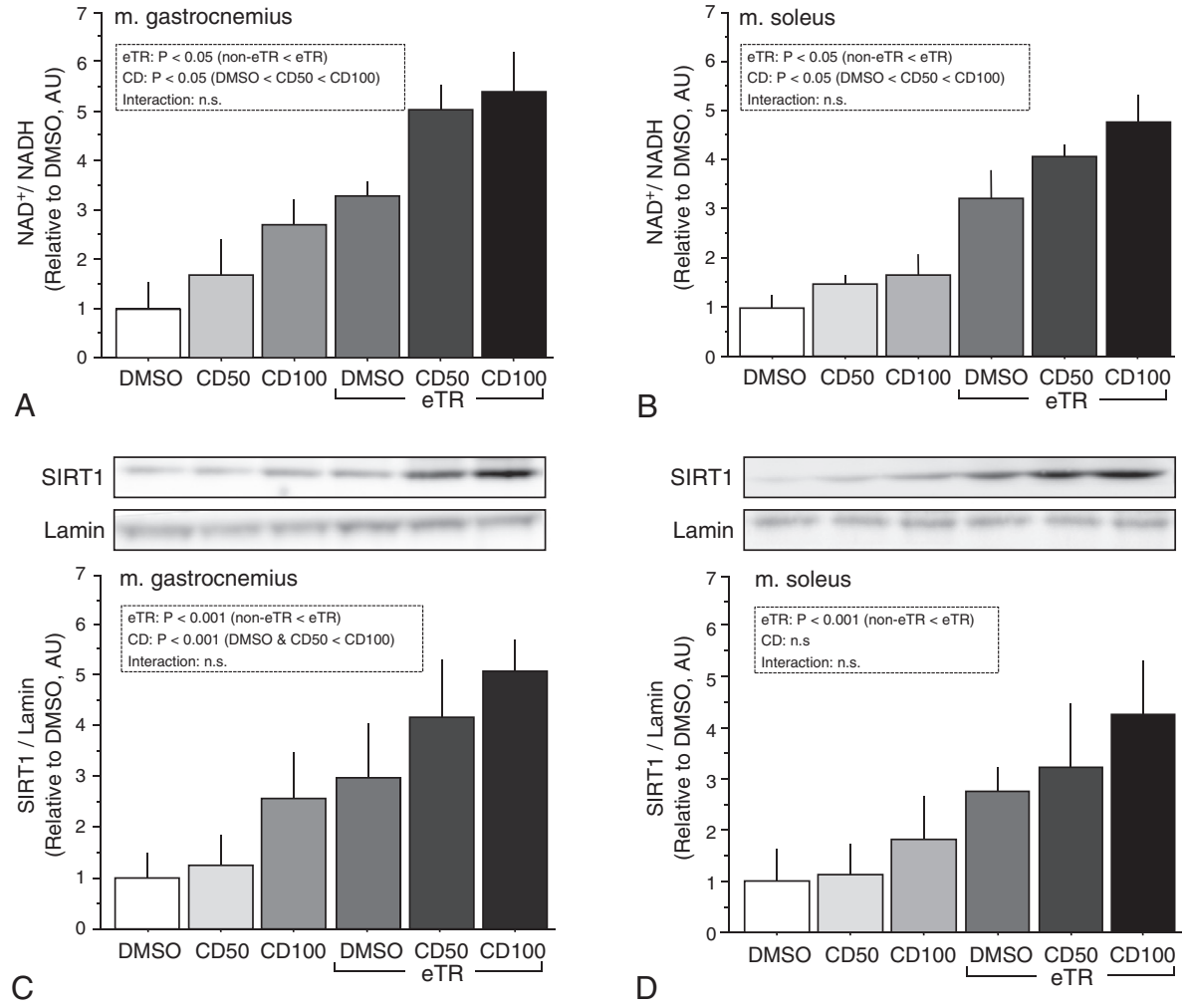


Fig. 5 – NAD^+/NADH ratio and SIRT1 protein expression in skeletal muscle.Value are mean ± SD (n = 6 in each group) DMSO = control without endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. Two-way ANOVA revealed the main effect for eTR (P < 0.001) and curcumin treatment (P < 0.001) for NAD^+/NADH ratio in Gas and Sol (A and B) with no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses of curcumin increased NAD^+/NADH ratio in Gas and Sol (A and B, P < 0.05). Additionally, two-way ANOVA revealed main effect for eTR (P < 0.001) for SIRT1 expression in Gas and Sol (C and D). On the other hand, there was a main effect of curcumin treatment only in Gas (C, P < 0.001) but not in Sol (D, n.s.).

81.78, P < 0.001). Furthermore, exercise resulted in an increase in Gas compared to non exercise (Fig. 7A, F = 211.26, P < 0.001) with no interaction between the two factors (F = 0.29, n.s.). We determined the downstream target proteins of cAMP-dependent protein kinase (PKA) by using the PKA inhibitor H89. Our results showed that curcumin increased the phosphorylation of CREB and LKB-1 in Gas (Fig. 7B and C, F = 64.12 for p-CREB and 16.21 for p-LKB-1, P < 0.001) and H89 abolished this effect (Fig. 7B and C, F = 101.38 for p-CREB and 41.35 for p-LKB-1, P < 0.001). These results suggested that curcumin increases the levels of cAMP as well as the phosphorylation of CREB and LKB-1 in skeletal muscle in addition to the effect of exercise.

4. Discussion

The physiological significance of mitochondrial biogenesis is enhancement of cellular oxidative phosphorylation, which improves the endurance of the organism. Pathways that regulate mitochondrial biogenesis have recently emerged as potential metabolic targets. Previous studies presented evidence that curcumin could induce mitochondrial biogenesis in the liver and brain [35,36]. Our data support the finding that curcumin upregulates the expression of mitochondrial markers COX-IV (Figs. 1A and 2A), and OXPHOS sub unit (Figs. 1B and C, 2B and C) as well as increases the mtDNA copy number (Fig. 3A) and CS activity (Fig. 3B). Furthermore, we demonstrated that eTR increases in the expression of COX-IV (Figs. 1A and 2A), OXPHOS subunit (Figs. 1B and 2B), mitochondrial DNA copy number (Fig. 3A), and CS activity (Fig. 3B) as predicted. To our knowledge, this study is the first to demonstrate increased mitochondrial biogenesis in rat skeletal muscle stimulated by a combination of curcumin and exercise. The effect of curcumin appeared less in Sol than that in Gas and this could be owing to differences in the recruitment pattern of lower limb muscles during rat swimming [37]. This evidence was strengthened by the previous study which showed that swimming exercise did not increase PGC-1α which is involved in regulation of mitochondrial biogenesis in soleus muscle [6]. The previous research suggested that the polyphenol resveratrol combined with exercise could regulated mitochondrial biogenesis through its antioxidant activity [38]. In fact, the increased mitochondrial capacity stimulated by physical exercise was actually ROS-dependent, and ROS generated in muscles during exhaustive activity was the stimulus that triggered mitochondrial biogenesis [39]. Curcumin, however, is a more powerful antioxidant than other polyphenols including resveratrol [23] and biomedical investigations of curcumin have provided evidence for a wide range of molecular and cellular activities, mostly related to redox reactions and signal transduction [40]. We therefore speculated that the bioactivity of curcumin in skeletal muscles would be superior to that of resveratrol and directed through the regulation of mitochondrial biogenesis. In this study, curcumin treatment increased the levels of markers of mitochondrial biogenesis in skeletal muscle in a similar fashion to combination treatment with endurance training, suggesting that curcumin treatment together with exercise plays a role in the regulation of mitochondrial biogenesis in skeletal muscle.

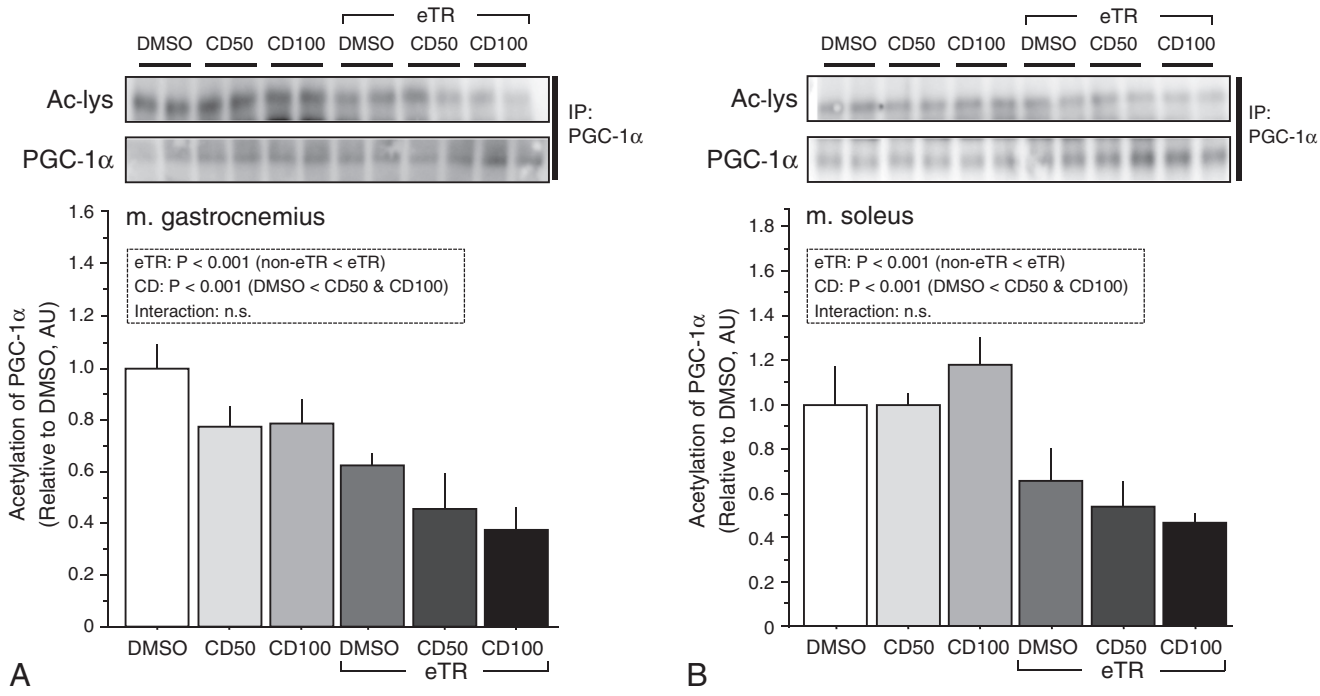


Fig. 6 – Acetylation of PGC-1α. In skeletal muscle.Value are mean ± SD (n = 6 in each group) DMSO = control without endurance exercise. CD 50 = curcumin 50 mg/kg-BW/day in DMSO. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. Two-way ANOVA revealed the main effect for eTR (P < 0.001) and curcumin treatment (P < 0.001) for acetylation of PGC-1α in Gas (A) and Sol (B) with no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, both doses of curcumin decreased acetylation of PGC-1α in Gas and Sol (P < 0.05).

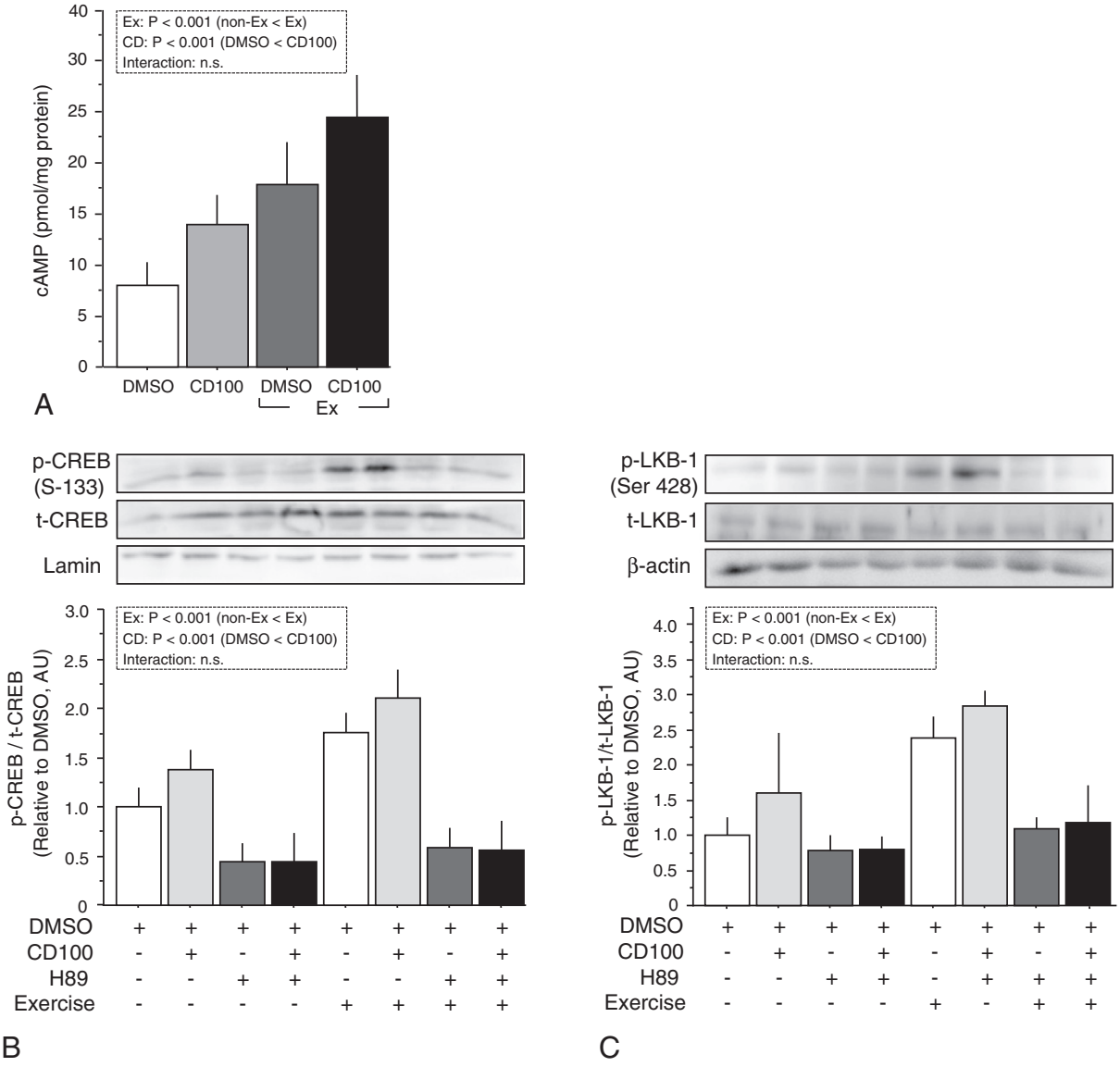


Fig. 7 – cAMP level (A) and CREB (B) and LKB-1 (C) phosphorylation in gastrocnemius skeletal muscle.Value are mean ± SD (n = 6 in each group) DMSO = control without exercise. CD 100 = curcumin 100 mg/kg-BW/day in DMSO. Ex = exercise. (A) Two-way ANOVA revealed the main effect for exercise (P < 0.001) and curcumin treatment (P < 0.001) for increased cAMP level in Gas with no interaction between two factors (n.s.). Post-hoc analysis revealed that compared to DMSO group, curcumin increased cAMP level in Gas (P < 0.05). (B) Two-way ANOVA revealed main effect for exercise (P < 0.001) and curcumin treatment (P < 0.001) for phosphorylation of CREB in Gas and H89 abolished this effect (P < 0.001). (C) Two-way ANOVA revealed main effect for exercise (P < 0.001) and curcumin treatment (P < 0.001) for phosphorylation of LKB-1 in Gas and H89 abolished this effect (P < 0.001).

Exercise is known to induce mitochondrial biogenesis in skeletal muscles and improve performance by increasing oxidative capacity [41,42] for which the enzyme AMPK serves as a metabolic sensor. Structurally, mammalian AMPK is a heterotrimer of three subunits: one catalytic (α) and two regulatory (β and γ) subunits [43,44]. In skeletal muscles, exercise can elevate the cellular [AMP]/[ATP] ratio in cells [45], thereby activating AMPK [46–48], which is a critical regulator of mitochondrial biogenesis in response to energy deprivation. A previous study demonstrated that curcumin treatment could increase the phosphorylation of AMPK and alleviate insulin resistance in skeletal muscles [31]. In adipocytes of C57/BL6 mice fed a high-fat diet, curcumin was shown to increase the phosphorylation of AMPK [49] suggesting that the AMPK pathway might be a target for curcumin in regulating mitochondrial biogenesis in skeletal muscles. Indeed, our results demonstrated that high doses of curcumin induced the phosphorylation of AMPK in Gas and Sol (Fig. 4A and B). In addition, in accordance with the results of many recent studies, our results confirmed that eTR increased the phosphorylation of AMPK in skeletal muscles and furthermore that curcumin co-treatment was additive to the effect of eTR (Fig. 4A and B). When the intracellular energy state changes because of exercise, an increase in the cellular NAD^+ concentration and the resulting interaction of AMPK and SIRT1 can induce the expression of proteins involved in

mitochondria biogenesis. In this study, curcumin was shown to increase the NAD^+/NADH ratio, resulting in an increase in SIRT1 expression (Fig. 5).

SIRT1-mediated PGC-1α deacetylation may be essential to activate mitochondrial biogenesis in skeletal muscles [50,51]. Thus, a number of studies have observed that SIRT1 levels of gene expression and protein increase in skeletal muscles in response to acute and chronic exercise, in parallel to an up-regulation of mitochondrial content. Our results demonstrated that curcumin treatment increased the cytosol NAD^+/NADH ratio followed by an increase in the expression of SIRT1 protein in the nuclear fraction in Gas (Fig. 5A and C). Furthermore, eTR also increased the NAD^+/NADH ratio and SIRT1 expression (Fig. 5). In addition, some polyphenols, including curcumin, have also been shown to activate SIRT1 directly or indirectly in a variety of models [52,53]. This has led to great interest in developing polyphenols to target the SIRT1-PGC-1α complex or related signaling pathways in order to regulate mitochondrial biogenesis in skeletal muscles, and mimic or potentiate the effect of eTR. One study has shown that the activation of SIRT1 by curcumin attenuates myocardial IR-induced mitochondrial oxidative damage [54]. However, the present study is the first, to our knowledge, to demonstrate increases in the expression of SIRT1 protein in both skeletal muscle types in response to curcumin treatment combined with eTR (Fig. 5C and D).

PGC-1α is a coactivator, involved in activating both nuclear and mitochondrial transcription, resulting in mitochondrial biogenesis and the up-regulation of genes involved in lipid metabolism and oxidative phosphorylation [55–57]. Various transcriptional factors including nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (Tfam) may be involved [58]. A previous study has shown that PGC-1α knockdown in skeletal muscle cells significantly reduced the expression of Tfam and COX-IV [59]. PGC-1α can also be regulated at the posttranslational level, for instance by acetylation [60]. It has been reported that polyphenol combined with exercise increase deacetylation PGC-1α [32]. We therefore assessed the acetylation/deacetylation state of PGC-1α in the muscles of animals treated with curcumin with or without eTR. The ratio of acetylated PGC-1α to total PGC-1α protein in the nucleus was significantly decreased by curcumin treatment combined with eTR in both Gas and Sol (Fig. 6) indicating that deacetylation of PGC-1α was facilitated by curcumin. Therefore, curcumin treatment had a significant impact on the state of PGC-1α and mitochondrial biogenesis when skeletal muscles were subjected to the repeated metabolic demands of eTR, and may have the potential to exert an additive effect together with eTR.

As discussed above, deacetylation of PGC-1α by SIRT1 is thought to be an important step in increasing PGC-1α activity, and SIRT1 protein expression increased with eTR and curcumin (Fig. 5C and D). Although the mechanisms presently remain unclear, nitric oxide synthase (NOS) may be involved in this effect. Recent studies have shown that curcumin treatment increases NOS enzyme activity in rat endothelium and increases the expression of eNOS and nNOS genes [61,62]. Another candidate is AMPK, since endurance exercise increases AMPK activity in skeletal muscles [29,30]. The results reported here regarding the increase in SIRT1 expression and NAD^+/NADH ratio in skeletal muscles following eTR in combination with curcumin treatment, may help explain the additive effects of the combined treatment *in vivo*. Our findings demonstrate the therapeutic potential of curcumin in the induction of mitochondrial biogenesis and function, which may be dependent on the presence of SIRT1 as well as on a cellular environment created by the continuous energy demands of repeated exercise. Similarly, the beneficial effects of both calorie restriction and the polyphenol resveratrol have been suggested to involve activation of SIRT1 and AMPK [63–65]. Thus, there is a dynamic interaction between these two pathways with AMPK activating SIRT1, probably through an indirect increase in cellular NAD^+ concentration [66]. According to the above results, the present study suggests that the effects of phosphorylation and activation of AMPK (caused by exercise and curcumin treatment) trigger an increase in the cellular NAD^+/NADH ratio, which activates SIRT1. In addition, AMPK also phosphorylates PGC-1α and primes it for subsequent deacetylation by SIRT1. The effect of AMPK and SIRT1 on the acetylation status of PGC-1α, and possibly other transcriptional regulators, subsequently improves mitochondrial biogenesis (Fig. 8). Since this is the first report showing the additive effect of curcumin and eTR on the phosphorylation of AMPK skeletal muscle, the precise mechanism remains unknown.

cAMP is a critical, tightly regulated second messenger involved in many intracellular processes and degraded by PDE. cAMP activates PKA, which, in turn, phosphorylates CREB and LKB-1 thereby regulating mitochondrial biogenesis through PGC-1α. Our results indicated that curcumin and exercise increased the levels of cAMP in Gas (Fig. 7A). This result confirms the results of a previous report, which demonstrated the potential of curcumin to increase cAMP levels [34,67]. Identification of cAMP as a target for curcumin in this present study might explain how curcumin mimics some aspects of exercise. To determine whether cAMP is required to regulate mitochondrial biogenesis, we used PKA inhibitor H89 and showed that the induced phosphorylation of CREB and LKB-1 (Fig. 7B and C) in Gas was abolished (Fig. 7B and C). This result indicates that cAMP is needed to regulate mitochondrial biogenesis in skeletal muscle and is in accordance with the results of a previous study, which showed that curcumin increased the phosphorylation of CREB in the brain [68] and of LKB-1 in skeletal muscle [31]. Activated CREB and LKB-1 have proven to be important regulators of the PGC-1α promoter [69,70]. The expression of PGC-1α was mediated by the activation of the transcription factor CREB, which requires phosphorylation by PKA to initiate mitochondrial biogenesis [71] while, LKB-1 regulated PGC-1α through the AMPK pathway [70]. These results suggest that the ability to increase the levels of cAMP plays an important role in the adaptation of mitochondrial biogenesis by curcumin either alone or combined with exercise (Fig. 8). Since this the first study to report on the effect of curcumin together with exercise on skeletal muscle, further investigation will be necessary to describe the molecular mechanisms and identify whether curcumin directly induces AMPK and SIRT1 to regulate mitochondrial biogenesis in skeletal muscle.

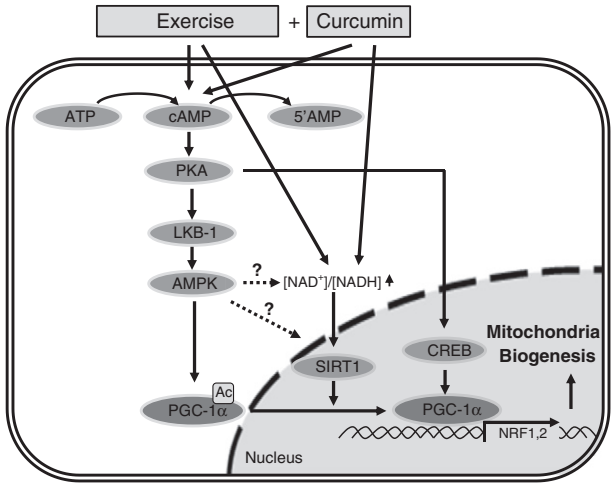


Fig. 8 – Molecular pathways involved in the effects of exercise and curcumin treatment on the mitochondrial biogenesis.Curcumin together with exercise increases cAMP level, which activates PKA and increase phosphorylation CREB and LKB-1. Phosphorylation and activation of AMPK triggers an increase in the cellular NAD^+/NADH ratio, which activates SIRT1. In addition, AMPK phosphorylates PGC-1α and primes it for subsequent deacetylation by SIRT1. The effect of AMPK and SIRT1 on the acetylation status of PGC-1α, and potentially of other transcriptional regulators, subsequently improves mitochondrial biogenesis. AMPK, 5′ adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; eTR, endurance training; LKB-1, liver kinase B1; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrogen; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1; PKA, protein kinase A; SIRT1, sirtuin 1.

5. Conclusion

In conclusion, curcumin treatment combined with eTR activated AMPK and increased the NAD^+/NADH ratio in skeletal muscle to induce SIRT1 activation. Activation of AMPK and SIRT1 promoted the deacetylation of PGC-1α and enhanced the expression of the mitochondrial marker COX IV, CS enzyme activity, OXPHOS sub unit expression, and mtDNA copy number. Furthermore, curcumin increased the levels of cAMP and phosphorylation of CREB and LKB-1, which are involved in the regulation of mitochondrial biogenesis. Taken together, our data suggest that curcumin treatment enhances the effect of eTR on the regulation of mitochondrial biogenesis in skeletal muscles through an increase in the level of cAMP.

Author Contribution

R.H. and K.M. are responsible for conception and design of the research; R.H. and T.Y. performed the experiments; R.H. and K.M. analyzed the data; R.H., T,Y., R.I., Y.S. and K.M. interpreted the results of the experiments; R.H. and K.M. prepared the figures; R.H. and K.M. drafted the manuscript; R.H., T. Y., R.I., Y.S. and K.M. edited and revised the manuscript; K.M. approved the final version of the manuscript.

Funding

This research was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture (26282183, 15K12665, KM), and also by funding from Shibuya Science, Culture and Sport Foundation, Japan (KM). RH was supported by a scholarship from Directorate General of High Education, Indonesia, for the international collaboration.

Conflict of Interest

None.

REFERENCES

[1] Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, et al. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A 2003;100:8466–71.

[2] Joseph AM, Joanisse DR, Baillot RG, Hood DA. Mitochondrial dysregulation in the pathogenesis of diabetes: potential for mitochondrial biogenesis-mediated interventions. Exp Diabetes Res 2012;2012:642038.

[3] Holloszy JO. Regulation by exercise of skeletal muscle content of mitochondria and GLUT4. J Physiol Pharmacol 2008;59(Suppl. 7):5–18.

[4] Piantadosi CA, Carraway MS, Babiker A, Suliman HB. Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. Circ Res 2008;103:1232–40.

[5] Hock MB, Kralli A. Transcriptional control of mitochondrial biogenesis and function. Annu Rev Physiol 2009;71:177–203.

[6] Terada S, Tabata I. Effects of acute bouts of running and swimming exercise on PGC-1alpha protein expression in rat epitrochlearis and soleus muscle. Am J Physiol Endocrinol Metab 2004;286:208–16.

[7] Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, Tabata I. Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochem Biophys Res Commun 2002;296:350–4.

[8] Pilegaard H, Saltin B, Neufer PD. Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. J Physiol 2003;546:851–8.

[9] Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, et al. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J 2002;16:1879–86.

[10] Taylor EB, Lamb JD, Hurst RW, Chesser DG, Ellingson WJ, Greenwood LJ, et al. Endurance training increases skeletal muscle LKB1 and PGC-1alpha protein abundance: effects of time and intensity. Am J Physiol Endocrinol Metab 2005;289:960–8.

[11] Russell AP, Feilchenfeldt J, Schreiber S, Praz M, Crettenand A, Gobelet C, et al. Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and

peroxisome proliferator-activated receptor-alpha in skeletal muscle. Diabetes 2003;52:2874–81.
[12] Baur JA, Chen D, Chini EN, Chua K, Cohen HY, de Cabo R, et al. Dietary restriction: standing up for sirtuins. Science 2010;329: 1012–3 [author reply 3–4].
[13] Donmez G, Guarente L. Aging and disease: connections to sirtuins. Aging Cell 2010;9:285–90.
[14] Suwa M, Nakano H, Radak Z, Kumagai S. Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor gamma coactivator-1alpha protein expressions in rat skeletal muscle. Metabolism 2008;57:986–98.
[15] Ferrara N, Rinaldi B, Corbi G, Conti V, Stiuso P, Boccuti S, et al. Exercise training promotes SIRT1 activity in aged rats. Rejuvenation Res 2008;11:139–50.
[16] Suwa M, Nakano H, Radak Z, Kumagai S. Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor γ coactivator-1α protein expressions in rat skeletal muscle. Metabolism 2008;57:986–98.
[17] Bogacka I, Ukropcova B, McNeil M, Gimble JM, Smith SR. Structural and functional consequences of mitochondrial biogenesis in human adipocytes in vitro. J Clin Endocrinol Metab 2005;90:6650–6.
[18] Than TA, Lou H, Ji C, Win S, Kaplowitz N. Role of cAMP-responsive element-binding protein (CREB)-regulated transcription coactivator 3 (CRTC3) in the initiation of mitochondrial biogenesis and stress response in liver cells. J Biol Chem 2011;286:22047–54.
[19] Veeranki S, Hwang S-H, Sun T, Kim B, Kim L. LKB1 regulates development and the stress response in Dictyostelium. Dev Biol 2011;360:351–7.
[20] Palmer WK. Effect of exercise on cardiac cyclic AMP. Med Sci Sports Exerc 1988;20:525–30.
[21] Park S-J, Ahmad F, Philp A, Baar K, Williams T, Luo H, et al. Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. Cell 2012;148:421–33.
[22] Chowanadisai W, Bauerly KA, Tchaparian E, Wong A, Cortopassi GA, Rucker RB. Pyrroloquinoline quinone stimulates mitochondrial biogenesis through cAMP response element-binding protein phosphorylation and increased PGC-1alpha expression. J Biol Chem 2010;285:142–52.
[23] Aftab N, Vieira A. Antioxidant activities of curcumin and combinations of this curcuminoid with other phytochemicals. Phytother Res 2010;24:500–2.
[24] Eckert GP, Schiborr C, Hagl S, Abdel-Kader R, Muller WE, Rimbach G, et al. Curcumin prevents mitochondrial dysfunction in the brain of the senescence-accelerated mouse-prone 8. Neurochem Int 2013;62:595–602.
[25] Kawanaka K, Tabata I, Katsuta S, Higuchi M. Changes in insulin-stimulated glucose transport and GLUT-4 protein in rat skeletal muscle after training. J Appl Physiol 1997;83:43–7.
[26] Blough E, Dineen B, Esser K. Extraction of nuclear proteins from striated muscle tissue. Biotechniques 1999;26:202–4 [206].
[27] Furuichi Y, Sugiura T, Kato Y, Shimada Y, Masuda K. OCTN2 is associated with carnitine transport capacity of rat skeletal muscles. Acta Physiol 2010;200:57–64.
[28] Srere PA. [1] Citrate synthase: [EC 4.1.3.7. Citrate oxaloacetate-lyase (CoA-acetylating)]. In: John ML, editor. Meth EnzymolAcademic Press; 1969. p. 3–11.
[29] Stein SC, Woods A, Jones NA, Davison MD, Carling D. The regulation of AMP-activated protein kinase by phosphorylation. Biochem J 2000;3:437–43.
[30] Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A, et al. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. EMBO J 2005;24:1810–20.
[31] Na LX, Zhang YL, Li Y, Liu LY, Li R, Kong T, et al. Curcumin improves insulin resistance in skeletal muscle of rats. Nutr Metab Cardiovasc Dis 2011;21:526–33.
[32] Gurd BJ. Deacetylation of PGC-1alpha by SIRT1: importance for skeletal muscle function and exercise-induced mitochondrial biogenesis. Appl Physiol Nutr Metab 2011;36: 589–97.
[33] Ohyama K, Nogusa Y, Suzuki K, Shinoda K, Kajimura S, Bannai M. A combination of exercise and capsinoid supplementation additively suppresses diet-induced obesity by increasing energy expenditure in mice. Am J Physiol Endocrinol Metab 2015;308:315–23.
[34] Abusnina A, Keravis T, Lugnier C. D020 The polyphenol curcumin inhibits in vitro angiogenesis and cyclic nucleotide phosphodiesterases (PDEs) activities similarly to PDE inhibitors. Arch Cardiovasc Dis 2009;102(Supplement 1):S43.
[35] Liu L, Zhang W, Wang L, Li Y, Tan B, Lu X, et al. Curcumin prevents cerebral ischemia reperfusion injury via increase of mitochondrial biogenesis. Neurochem Res 2014;39:1322–31.
[36] Kuo JJ, Chang HH, Tsai TH, Lee TY. Curcumin ameliorates mitochondrial dysfunction associated with inhibition of gluconeogenesis in free fatty acid-mediated hepatic lipoapoptosis. Int J Mol Med 2012;30:643–9.
[37] Roy RR, Hutchison DL, Pierotti DJ, Hodgson JA, Edgerton VR. EMG patterns of rat ankle extensors and flexors during treadmill locomotion and swimming. J Appl Physiol 1985;70: 2522–9.
[38] Menzies KJ, Singh K, Saleem A, Hood DA. Sirtuin 1-mediated effects of exercise and resveratrol on mitochondrial biogenesis. J Biol Chem 2013;288:6968–79.
[39] Viña J, Gomez-Cabrera MC, Borras C, Froio T, Sanchis-Gomar F, Martinez-Bello VE, et al. Mitochondrial biogenesis in exercise and in ageing. Adv Drug Deliv Rev 2009;61:1369–74.
[40] Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci 2008;65:1631–52.
[41] Hood DA. Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. J Appl Physiol 1985;90:1137–57.
[42] Williams RS, Salmons S, Newsholme EA, Kaufman RE, Mellor J. Regulation of nuclear and mitochondrial gene expression by contractile activity in skeletal muscle. J Biol Chem 1986; 261:376–80.
[43] Cheung PC, Salt IP, Davies SP, Hardie DG, Carling D. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. Biochem J 2000;346:659–69.
[44] Stapleton D, Woollatt E, Mitchelhill KI, Nicholl JK, Fernandez CS, Michell BJ, et al. AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location. FEBS Lett 1997;409: 452–6.
[45] Chen ZP, Stephens TJ, Murthy S, Canny BJ, Hargreaves M, Witters LA, et al. Effect of exercise intensity on skeletal muscle AMPK signaling in humans. Diabetes 2003;52: 2205–12.
[46] Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem 1998;67:821–55.
[47] Hardie DG, Hawley SA. AMP-activated protein kinase: the energy charge hypothesis revisited. BioEssays 2001;23: 1112–9.
[48] Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, et al. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. Proc Natl Acad Sci U S A 2002;99:15983–7.
[49] Ejaz A, Wu D, Kwan P, Meydani M. Curcumin inhibits adipogenesis in 3T3-L1 adipocytes and angiogenesis and obesity in C57/BL mice. J Nutr 2009;139: 919–25.
[50] Rodgers JT, Lerin C, Gerhart-Hines Z, Puigserver P. Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. FEBS Lett 2008;582:46–53.

[51] Price Nathan L, Gomes Ana P, Ling Alvin JY, Duarte Filipe V, Martin-Montalvo A, North Brian J, et al. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell Metab 2012;15:675–90.
[52] Queen BL, Tollefsbol TO. Polyphenols and aging. Curr Aging Sci 2010;3:34–42.
[53] Chung S, Yao H, Caito S, Hwang JW, Arunachalam G, Rahman I. Regulation of SIRT1 in cellular functions: role of polyphenols. Arch Biochem Biophys 2010;501:79–90.
[54] Yang Y, Duan W, Lin Y, Yi W, Liang Z, Yan J, et al. SIRT1 activation by curcumin pretreatment attenuates mitochondrial oxidative damage induced by myocardial ischemia reperfusion injury. Free Radic Biol Med 2013;65: 667–79.
[55] Benton CR, Nickerson JG, Lally J, Han XX, Holloway GP, Glatz JF, et al. Modest PGC-1alpha overexpression in muscle in vivo is sufficient to increase insulin sensitivity and palmitate oxidation in subsarcolemmal, not intermyofibrillar, mitochondria. J Biol Chem 2008;283:4228–40.
[56] Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, et al. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 2002;418: 797–801.
[57] Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 1999;98:115–24.
[58] Kuo JJ, Chang HH, Tsai TH, Lee TY. Positive effect of curcumin on inflammation and mitochondrial dysfunction in obese mice with liver steatosis. Int J Mol Med 2012;30: 673–9.
[59] Uguccioni G, Hood DA. The importance of PGC-1alpha in contractile activity-induced mitochondrial adaptations. Am J Physiol Endocrinol Metab 2011;300:361–71.
[60] Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 2005; 434:113–8.
[61] Abdel Aziz MT, Motawi T, Rezq A, Mostafa T, Fouad HH, Ahmed HH, et al. Effects of a water-soluble curcumin protein conjugate vs. pure curcumin in a diabetic model of erectile dysfunction. J Sex Med 2012;9:1815–33.
[62] Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, et al. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 2005;310:314–7.
[63] Boily G, He XH, Pearce B, Jardine K, McBurney MW. SirT1-null mice develop tumors at normal rates but are poorly protected by resveratrol. Oncogene 2009;28:2882–93.
[64] Boily G, Seifert EL, Bevilacqua L, He XH, Sabourin G, Estey C, et al. SirT1 regulates energy metabolism and response to caloric restriction in mice. PLoS One 2008;3:e1759.
[65] Um JH, Park SJ, Kang H, Yang S, Foretz M, McBurney MW, et al. AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol. Diabetes 2010;59:554–63.
[66] Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, et al. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 2009;458:1056–60.
[67] Rouse M, Younes A, Egan JM. Resveratrol and curcumin enhance pancreatic beta-cell function by inhibiting phosphodiesterase activity. J Endocrinol 2014;223:107–17.
[68] Nam SM, Choi JH, Yoo DY, Kim W, Jung HY, Kim JW, et al. Effects of curcumin (*Curcuma longa*) on learning and spatial memory as well as cell proliferation and neuroblast differentiation in adult and aged mice by upregulating brain-derived neurotrophic factor and CREB signaling. J Med Food 2014;17:641–9.
[69] Fernandez-Marcos PJ, Auwerx J. Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis. Am J Clin Nutr 2011;93:884–90.
[70] Koh HJ, Brandauer J, Goodyear LJ. LKB1 and AMPK and the regulation of skeletal muscle metabolism. Curr Opin Clin Nutr Metab Care 2008;11:227–32.
[71] Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev 2004;18:357–68.