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Mitogen-activated protein kinase kinases promote mitochondrial biogenesis in part through inducing peroxisome proliferator-activated receptor γ coactivator-1β expression

Minghui Gao [a,1,2], Junjian Wang [a,1,3], Na Lu [a,1], Fang Fang [a], Jinsong Liu [a,*], Chi-Wai Wong [b,**]

[a] Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, 510530 China
[b] NeuMed Pharmaceuticals Limited, Hong Kong

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ABSTRACT

Growth factor activates mitogen-activated protein kinase kinases to promote cell growth. Mitochondrial biogenesis is an integral part of cell growth. How growth factor regulates mitochondrial biogenesis is not fully understood. In this study, we found that mitochondrial mass was specifically reduced upon serum starvation and induced upon re-feeding with serum. Using mitogen-activated protein kinase kinases inhibitor U0126, we found that the mRNA expression levels of ATP synthase, cytochrome-C, mitochondrial transcription factor A, and mitofusin 2 were reduced. Since the transcriptional levels of these genes are under the control of peroxisome proliferator-activated receptor γ coactivator-1α and -1β (PGC-1α and PGC-1β), we examined and found that only the mRNA and protein levels of PGC-1β were suppressed. Importantly, over-expression of PGC-1β partially reversed the reduction of mitochondrial mass upon U0126 treatment. Thus, we conclude that mitogen-activated protein kinase kinases direct mitochondrial biogenesis through selectively inducing PGC-1β expression.

1. Introduction

Growth factor activation of receptor tyrosine kinase, which eventually activates mitogen-activated protein kinase kinases (MEK1/2) and their down-stream targets extracellular signal-regulated kinases (ERK1/2), plays a central role in the regulation of cell growth and proliferation [1]. Mutations or amplifications of these signaling molecules can contribute to the development of a broad spectrum of human cancers [2]. Suppression of ERK activation by specific inhibitors, dominant-negative forms of ERK, as well as ERK antisense nucleotides have been shown to inhibit cell proliferation and induce apoptosis; furthermore, MEK inhibitors significantly altered the abilities of these cells to proliferate in response to growth factor stimulation [2].

The roles of mitochondrial biogenesis and function in cell growth and proliferation are actively being investigated. In order to promote cell growth and division, the MEK–ERK cascade needs to coordinately regulate protein, nucleic acid, and lipid biosynthesis for genome and organelle duplications. All of these processes consume high levels of energy; thus, mitochondrial biogenesis and energy metabolism must also be highly coordinated by the MEK–ERK cascade. Recent studies have shown that modulating ERK1/2 activities alters mitochondrial function [3,4]. However, how MEK–ERK cascade governs mitochondrial biogenesis remains undefined.

Estrogen-related receptors α and γ (ERRα and ERRγ) together with their coactivators peroxisome proliferator-activated receptor γ coactivator-1α and -1β (PGC-1α and PGC-1β) are key regulators of mitochondria biogenesis and function [5,6]. These transcriptional regulators bind to response elements located on the promoters of their target genes such as ATP synthase (ATP5b), cytochrome-C (cyt-C), mitochondrial transcription factor A (Tfam), and mitofusin 2 (Mfn2) to guide the expressions of these mitochondrial enzymes and regulators [7]. In this study, we investigated whether ERRα, ERRγ, PGC-1α, and PGC-1β participate in communicating the signal from

Abbreviations: ATP5b, ATP synthase; cyt-C, cytochrome-C; ERRα and ERRγ, estrogen-related receptors α and γ; ERK1/2, extracellular signal-regulated kinases; Tfam, mitochondrial transcription factor A; Mfn2, mitofusin 2; MEK1/2, mitogen-activated protein kinase kinases; PGC-1α and PGC-1β, peroxisome proliferator-activated receptor γ coactivator-1α and -1β

* Correspondence to: Jinsong Liu, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, 190 Kai Yuan Avenue, Science Park, Guangzhou, 510530 China. Tel.: +86 20 3201 5317.
** Correspondence to: Chi-Wai Wong, NeuMed Pharmaceuticals Limited, Hong Kong. Tel.: +852 6748 7218.
E-mail addresses: liu_jinsong@gibh.ac.cn (J. Liu), wongcw123456@yahoo.com (C.-W. Wong).
[1] These authors contributed equally to this study.
[2] Present address: Room 514, 5/F, Choh-MingLi Basic Medical Sciences Building, The Chinese University of Hong Kong, Shatin, Hong Kong.
[3] Present address: Room 1400 Research III Building, UC Davis Cancer Center/Basic Sciences, 4645 2nd Avenue, Sacramento, CA 95817, USA.

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the MEK–ERK cascade to govern mitochondrial biogenesis. We found that the mRNA expression level of PGC-1β was specifically induced by the MEK–ERK cascade.

2. Material and methods

2.1. Cell culture

Human non-small cell lung cancer (NSCLC) A549 cells were purchased from American Type Culture Collection (ATCC) and cultured in RIPM1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone) at 37 °C in 5% CO_2.

2.2. Confocal laser scanning microscopy of mitochondrial mass

A549 cells were seeded onto coverslip in 6-well plates and cultured overnight, after starvation or re-feeding treatment, cells were incubated in pre-warmed serum free medium with 400 nM Mitotracker Green FM and 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 20 min in the dark. After staining, cells were washed four times with cold phosphate-buffered saline (PBS) and mounted on glass slides. Images were subsequently taken with a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany).

2.3. Mitochondrial mass measurement

Mitotracker green (Invitrogen) was added and quantified as described [8]. Briefly, cells were incubated in serum free medium (pre-warmed to 37 °C) with 150 nM Mitotracker Green FM for 20 min in the dark. After staining, cells were washed twice with cold phosphate-buffered saline (PBS) and suspended in 200 μl PBS. Subsequently, cells were analyzed on a flow cytometer (FAC-SCalibur, BD Biosciences) with excitation at 490 nm and emission at 516 nm. Data were processed by using the CellQuest program (BD Biosciences). Assays done in triplicate were repeated at least three times.

2.4. Cellular ATP level measurement

Total cellular ATP level was measured by ATPlite-glo (PerkinElmer) following the manufacture's protocol using VERITAS™ Microplate luminometer (Turner Biosystems) as described [9].

2.5. Quantitative real-time PCR

Total RNA extraction, first-strand cDNA generation, and quantitative real-time PCR analysis were performed as described [10]. Relative gene expression was normalized to 18 S rRNA levels. Primer sequences and real-time PCR conditions are listed in Table S1 of the supplementary data.

2.6. Western blot analysis

Cell extracts were made in RIPA buffer (50 mM Tris pH7.5, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10 μg/ml Aprotinin) and whole cell extracts (50–75 μg of protein) were treated with SDS sample buffer, boiled for 5 min, and subjected to SDS–PAGE and Western analysis by ECL Western Blotting System (Amersham Pharmacia, UK). Membranes were incubated with rabbit anti-ERRα, -ERRγ, -PGC-1α, and -PGC-1β antibodies [11,12], or mouse anti-β-actin antibody (Boster) followed by horseradish peroxidase-conjugated secondary antibody (Amersham) and developed with ECL reagent (Amersham).

2.7. Plasmids and transient transfection

A pGL3-PGC-1β-promoter reporter plasmid was cloned by PCR amplification of its 2 kb genomic region upstream of the transcriptional start site into a pGL3-luciferase vector (Promega). Transient transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For luciferase reporter assays, cells at 85–95% confluency in 96-well plates were cotransfected with reporter plasmids (25 ng/well) and *Renilla* luciferase (3 ng/well) as an internal control for transfection efficiency. Six hours after transfection, cells were starved in different amount of FBS or treated with U0126 for 24 h. Luciferase activity was measured as described [13,14].

2.8. Over-expression by transient transfection

A pcDNA3.1-PGC-1β-expression plasmid was cloned by PCR amplification, MEK1 and MEK2 plasmids were gifts from Dr. Sylvain Meloche [15]. Cells were over-expressed with pcDNA3.1 or expression plasmids using Lipofectamine 2000 (Invitrogen).

2.9. Statistical analysis

Data are presented as mean ± SE and analyzed by a variance test (ANOVA). Asterisks indicate significant differences: $^{*}P<0.05$, $^{**}P<0.01$.

3. Results

3.1. Growth factor signaling and mitochondrial biogenesis

In order to address the importance of mitochondrial biogenesis in mediating growth factor stimulated cell proliferation, we first monitored the changes in mitochondrial mass in human non-small cell lung cancer A549 cells that were serum-starved and then re-fed with serum. Using Mitotracker Green, a fluorescence dye that binds to mitochondrion independent of its potential, we found by confocal microscopy that the amount of stained mitochondria reduced upon serum starvation but rebound partially upon serum re-fed (Fig. 1A). In order to quantify the changes, we starved cells in different amounts of serum to induce cell cycle arrest (supplemental data Fig. S1) followed by fluorescence-assisted cell sorting analysis (supplemental data Fig. S2). We found that serum starvation quantitatively reduced mitochondrial mass and the decrease was negatively correlated to the amount of serum supplemented (Fig. 1B). We then asked how quickly mitochondrial biogenesis would increase upon growth factor stimulation in starved cells re-fed with 10% FBS. We found that mitochondrial mass increased gradually over time as cells were released from cell cycle arrest (Fig. 1C). Similar observations were obtained using another lung cancer cell line 95D and a colon cancer cell line HCT-116 (supplemental data Fig. S3).

Besides quantifying mitochondrial mass by fluorescence dye staining, we also measured the mRNA expression levels of several nuclear-encoded mitochondrial regulatory factors such as Mfn2 and Tfam as well as respiratory chain enzymatic components such as cyt-C and ATP5b. Correspondingly, the mRNA expression levels of these genes, which serve as markers to more globally reflect the changes in mitochondrial biogenesis and function, were all reduced by serum starvation and stimulated by re-feeding with serum (Fig. 1D). Additionally, we checked the mRNA expression levels of several mitochondrial DNA-encoded respiratory chain enzymatic components such as cytochrome b (MT-CYB), cytochrome c oxidase subunit I (MT-COI), and ATP synthase 6 (MT-ATP6) and found similar modes of regulation (Fig. 1E).