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# Resistin destroys mitochondrial biogenesis by inhibiting the PGC-1α/NRF1/TFAM signaling pathway

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ABSTRACT

Mitochondrial biogenesis deficits in neuronal cells are associated with the pathological progression of neurodegenerative diseases. Resistin, a secretory adipocytokine, possesses multiple physiological functions in diverse cells and tissues. However, the effects of resistin on mitochondrial biogenesis in neuronal cells are still elusive. In the current study, we found that resistin caused a sustainable decrease in mitochondrial contents, including mitochondrial DNA/nuclear DNA ratio (mtDNA/nDNA), mitochondrial mass, cytochrome b protein expression, and cytochrome c oxidase activity, which were correlated with "loss of mitochondrial function" including reduced mitochondrial respiration rate and ATP production in human SH-SY5Y neuronal cells. Indeed, resistin treatment destroyed the expression of peroxisome proliferator activator receptor gamma-coactivator 1α (PGC-1α), a master regulator of mitochondrial biogenesis, as well as its downstream target genes including nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM). Notably, overexpression of PGC-1α could completely rescue mitochondrial biogenesis and mitochondrial deficits induced by resistin. Mechanistically, inhibition of 5′-adenosine monophosphate-activated protein kinase (AMPK) was shown to mediate the inhibitory effects of resistin on mitochondrial biogenesis.



## 1. Introduction

Mitochondria, essential and multifunctional organelles in neurons, have been reported to assume a critical role in numerous physiological processes, including cellular respiration, energy metabolism, and apoptosis [1]. Mitochondrial biogenesis plays a key role in maintaining mitochondrial homeostasis and meeting the demands of neuronal cells [2]. Impairment of mitochondrial biogenesis is a potential contributor to mitochondrial dysfunction and plays important roles in the pathological progression of several neurodegenerative diseases [3]. For example, reduced mitochondrial contents, including mitochondrial DNA (mDNA) and ATP production associated with destroyed PGC-1α/NRF1/TFAM signaling pathways, have been found in neuronal cells of Alzheimer's disease [4]. Efforts have been made to investigate neurological disorders associated with mitochondrial function, however, the underlying molecular mechanisms of impaired mitochondrial biogenesis in neurodegenerative diseases remain elusive. Identification of neurotoxins which could induce impairment of mitochondrial biogenesis in neuronal cells is beneficial for exploring novel therapeutic approaches for the treatment of neurodegenerative diseases.

Resistin is an important hormone characterized by the presence of 10–11 cysteine residues [5]. It is highly expressed in adipocytes in rodents, and in peripheral blood mononuclear cells (PBMC) and macrophages in human being [6]. Resistin has been associated with several diseases, including type 2 diabetes mellitus, cardiovascular diseases, and neurodegenerative diseases [7]. Also, various physiological functions of resistin in diverse tissues and organs have been reported in previous studies. For example, increased levels of circulating resistin have been found in obese mice. Treatment with recombinant resistin induced hepatic insulin resistance in mice [8]. Additionally, resistin mediates the inflammatory response by increasing the expression of proinflammatory cytokines such as TNF-α, IL-6, and IL-12 in macrophages via the nuclear factor-κB (NF-κB) pathway [9]. Interestingly, administration of resistin has been shown to prevent the activation of 5′-adenosine monophosphate-activated protein kinase (AMPK) by reducing its phosphorylation in several tissues, including liver and skeletal muscle [10]. In

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contrast, blockage of resistin increases phosphorylation of AMPK [11]. AMPK has been considered as a master modulator of cell metabolism. Importantly, AMPK plays a key role in regulating mitochondrial biogenesis by promoting the expressions of PGC1-α and NRF1 [12]. The inhibitory effects of resistin on AMPK lead us to speculate that resistin might negatively impact mitochondrial biogenesis. In the current study, we aimed to investigate the effects of resistin on mitochondrial content and mitochondrial biogenesis signaling pathways in human SHSY5Y cells.

## 2. Materials and methods

### 2.1. Cell culture and treatment

Human neuronal SH-SY5Y cells were commercially obtained from ATCC, USA. Cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37 °C under a 5% $CO_2$ humidified atmosphere. Human SH-SY5Y cells were treated with resistin 5, 10, 20 ng/ml for 48 h.

### 2.2. Measurement of mitochondrial DNA/nuclear DNA (mtDNA/nDNA)

The ratio of mtDNA to nDNA (mtDNA/nDNA) in human SH-SY5Y cells was measured by real-time PCR analysis. Briefly, total DNA was extracted from SH-SY5Y cells using a commercial DNA extraction kit. Gene expression of a subunit of human electron transport chain ND1 was used to represent mtDNA, and gene expression of 18S ribosomal RNA was used to represent nDNA. Real-time PCR experiments were carried out on the LightCycler® 96 Real-Time PCR System using the Taqman method. The following primers were used in this study: ND1, (F): 5′-ATGGCCAACCTCCTACTCCT-3′; (R): 5′-GCGGTGATGTAGAGGGTGAT-3′; 18S, (F): 5′-ACGGACCAGAGCG AAAGCA-3′; (R): 5′-GACATCTAAGGGCATCACAGAC-3′.

### 2.3. Mitochondria staining with MitoTracker red

Mitochondrial mass in human SH-SY5Y cells was determined using mitochondrial staining with the dye Mitotracker Red (Life Technologies, USA). After the necessary transfection and treatment, human SH-SY5Y cells were incubated with 20 nM MitoTracker Red for 20 min. Nuclei of human SH-SY5Y cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min at RT. Fluorescent signals were visualized at 100 × oil immersion using a Zeiss fluorescence microscope. Eighty individual cells were randomly selected to calculate average integrated optical intensity.

### 2.4. Evaluation of intracellular ATP production

Total levels of ATP in human SH-SY5Y cells were measured using a convenient bioluminescence assay with an ATP Determination Kit (Thermo Fisher Scientific, USA). After the necessary transfection and treatment, human SH-SY5Y cells were lysed with cell lysis buffer. After centrifugation at 12000 × g for 15 min at 4 °C, 100 μl supernatant was collected and mixed with an equal amount of assay buffer containing luciferin/luciferase reagent. Luminescence was monitored at 560 nm using a luminometer.

### 2.5. Measurement of cytochrome c oxidase activity

Cytochrome c oxidase activity in SH-SY5Y cells was examined using a commercial cytochrome oxidase Microplate Assay Kit from Abcam, USA. After the necessary transfection and treatment, human SH-SY5Y cells were lysed with cell lysis buffer. After centrifugation at 12000 × g for 15 min at 4 °C, 10 μl supernatant was collected and mixed with an equal amount of assay buffer in 96-well plates. A 30-min kinetic program with a 30-sec interval was performed to examine cytochrome c oxidase activity.

### 2.6. Real-time polymerase chain reaction (PCR)

RNA was isolated from human SH-SY5Y cells using Trizol reagent (Life Technologies, USA). One μg purified RNA was used to make cDNA using an iScript RT-PCR Kit (Bio-Rad, USA). Two μl reaction product was used for real-time PCR analysis on a LightCycler® 96 Real-Time PCR System using the Taqman method. The conditions for real-time PCR condition were: denaturation (98 °C, 5 min), 40 cycles (98 °C for 30 s, 72 °C for 1 min). Expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used in this study: PGC-1α (F, 5′-CAATGAATGCAGCGGTCTTA-3′; R, 5′-ACGTCTTTGTGGCTTTTGCT-3′); NRF 1 (F, 5′-CTACTCGTGTGGGACAGCAA-3′; R, 5′-AATTCCGTC-GATGGTGAGAG-3′); TFAM (F, 5′-GGCACAGGAAACCAGTTAGG-3′; R, 5′-CAGAACACCGTGGCTTCTAC-3′); GAPDH (F, 5′-CCACATCGCTCA-GACACCAT-3′; R, 5′-CCAGGCGCCCAATACG-3′).

### 2.7. Western blot analysis

Human SH-SY5Y cells were harvested and lysed in cell lysis buffer (Cell Signaling Technology, USA) with protease and phosphatase inhibitor cocktails. Protein concentrations were measured using a BCA assay (Thermo Fisher Scientific, USA). Twenty μg total protein was separated by 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad, USA). After blocking with 5% non-fat milk for 2 h at RT, membranes were probed with primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated IgG secondary antibody. Signals were detected by a chemiluminescence detection kit (GE Healthcare, USA).

### 2.8. Determination of mitochondrial respiration rate

$O_2$ consumption of SH-SY5Y cells was measured using a commercial respirometer equipped with a Peltier thermostat and electromagnetic stirrer. After treatment with resistin for 48 h, $5 \times 10^6$ SH-SY5Y cells were collected and put in a glass chamber equilibrated in ambient room air with continuous stirring at the speed of 800 rpm for 10 min. The oxygen consumption was detected at 2 S intervals and the recording was stopped after stabilization of the $O_2$ consumption.

### 2.9. Statistical analysis

All experimental variables are expressed as means ± SEM. Experiments were repeated at least three times. Statistical significance was determined using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Resistin treatment causes impairment of mitochondrial biogenesis in neuronal cells

Firstly, we investigated the effects of resistin on mitochondrial content. An increase in the amount of mitochondrial DNA (mtDNA) is an important biomarker of mitochondrial biogenesis. Our results indicate that resistin treatment (10 ng/ml) significantly decreased the ratio of mtDNA to nDNA (mtDNA/nDNA) (Fig. 1A). We further evaluated mitochondrial mass in SHSY5Y cells in response to resistin treatment using MitoTracker Red staining. Fluorescence

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