Mitochondrial Dysfunction Enhances Lipolysis and Intracellular Lipid Accumulation in 3T3-L1 Adipocytes

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***Abstract*—Adipose tissue is one of the important peripheral tissues that regulate the whole-body homeostasis. Metabolic imbalance of energy productions and impaired oxidative phosphorylation in this target tissue may lead to mitochondrial dysfunction. However, it is currently unknown, what is the effect of mitochondrial dysfunctions in adipocytes on the cellular lipolysis activities and intracellular lipid accumulations. In this study, we determined the direct effects of mitochondrial dysfunction on the lipolysis activity and relative distribution of lipids in adipocytes. The induction of mitochondrial dysfunctions in adipocytes was performed with the treatment of two common mitochondrial respiratory inhibitors, antimycin A (Complex III) and oligomycin (ATP synthase) on 3T3-L1 adipocytes. We found that in the presence and absence of insulin, both respiratory inhibitors significantly reduced intracellular ATP concentrations within adipocytes. Furthermore, both drug treatments resulted in the significant elevation of free fatty acids and glycerol release into the media compared to control. The treated cells were also found to exhibit an irregular intracellular accumulation of lipid droplets. Our result demonstrated that lipolysis activity, and abnormal intracellular lipid accumulations were up-regulated in the event of mitochondrial dysfunctions in adipocytes, warranting further research are required for studying mechanisms underlying these metabolic impairments.**

***Index Terms*—3T3-L1 Adipocytes, Mitochondria, Lipolysis, Fatty Acids, Insulin Resistance, Type 2 Diabetes**

I. Introduction

Mitochondrion is the heart of the cell that can act as a powerhouse for energy production in the form of adenosine triphosphate (ATP). Energy stored in the fatty acids, glucose and amino acids are converted into this chemical energy. ATP is required for many cellular processes, including DNA, RNA and protein synthesis as well as maintenance of ion gradients across membranes. As metabolic regulations are largely dependent on mitochondria, it is not surprising to know the direct mechanisms of cell homeostasis, which utilize nutrient and energy generation, are the vital components that need to be looked out in diagnosing various metabolic disorders such as diabetes.

Correspondingly, in the following years; there are accumulating evidences on the roles of mitochondrial dysfunction in the pathogenesis of insulin resistance and type 2 diabetes. Numerous findings indicate prominent mitochondrial dysfunction in the skeletal muscle and adipose tissue in patients with insulin resistance or type 2 diabetes [1], [2]. Functional defects in mitochondrial functions in adipose tissues may result in the impaired glucose homeostasis. However, the pertinent role of mitochondrial dysfunctions in the electron transport chain (ETC) of adipocytes on the cellular lipolysis activity and lipid distributions remains elusive.

In this study, we observed the effects of both mitochondrial respiratory inhibitors antimycin A and oligomycin on the intracellular ATP contents of 3T3-L1 adipocytes. We also determined the activity of lipolysis in the event of mitochondrial dysfunctions in the treated cell. Both free fatty acids and glycerol release into the media were found to be up-regulated. The abnormal intracellular accumulations of lipid droplets also have been profoundly observed after treatment with these mitochondrial inhibitors.

II. Materials and Methodology

A. Cell Culture and Treatment

3T3-L1 pre-adipocytes were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of fetal calf serum and 1% of antibiotics. The cells were sub-cultured every three days before the culture becomes fully confluent, which are about 70%-80% of sub-confluent culture. The standard seeding density used was 2-4 × 10,000 cells /cm^2 and seeded in a 75cm2 flask. The cells were incubated at 37 ℃ in the humidified atmosphere of 5% CO_2. The 3T3-L1 pre-adipocytes were

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differentiated into adipocytes using a standard protocol [3]. The cells were incubated for 48 hours before undergo full differentiation. Later, cells were maintained in regular growth medium supplemented with adipogenic cocktail containing MDI (0.5mM 3-isobutyl-1-methyl-xanthine (IBMX), 0.25 µM dexamethasone and 1 µg/mL insulin) for 2 days. Induction of mitochondrial dysfunction in 3T3-L1 adipocytes was performed using two common mitochondrial respiratory inhibitors, oligomycin (ATP synthase inhibitor) and antimycin A (Complex III inhibitor). To affect the integrity of mitochondrial ETC, differentiated 3T3-L1 adipocytes were treated with the list of serial concentrations for 8 hours and the highest concentration that did not affect viability of the cells was chosen. The cells without mitochondrial inhibitors treatment were used as controls.

B. *Quantification of Cell Viability via MTT Assay*

MTT assay is a standard colorimetric assay for measuring cellular proliferation (cell growth). Yellow 3 - (4, 5-Dimethylthiazol-2-yl)-2-5 diphenyltetrazolium bromide (MTT) was reduced to purple formazan in the mitochondria of the living cell. Briefly, phosphate buffer saline (PBS) was used to dissolve 5mg/mL MTT before filtered through 0.2 µM micro-filter and will be stored at 4 ℃. 3T3-L1 adipocytes need to be washed out with PBS. 10 µL of MTT stock solution was prepared and subsequently added into each well and incubated for 3-4 hours at 37 °C. After that, 200 µL of dimethyl sulfoxide (DMSO) was transferred into each well to dissolve the insoluble purple formazan product into color solution. The absorbance of the colored solution was measured at the wavelength of 570 nm and reference wavelength of 630 nm using the ELISA plate reader.

C. *Measurement of Intracellular ATP Concentration*

Intracellular ATP concentrations were measured using a calorimetric ATP Assay Kit (ab83355; Abcam). The standard protocol of using this kit was based on the manufacturer's instruction [4].

D. *Measurement of Lipolysis Activity*

Lipolysis was evaluated by measuring the amount of glycerol and free fatty acids released into the media. Glycerol level was determined after 1 to 24 hours of oligomycin and antimycin A treatments following the manufacturer instructions. Free fatty acids were quantified after 3 hours of drugs treatment by using the Lipolysis As s ay KIT for Free Fatty Acids Detection (Zen -Bio Inc, Research Triangle Park, NC) according to the manufacturer's instructions.

E. *Quantification of Lipid Content by Oil Red O Assay*

Intracellular accumulations of lipid droplets were determined by oil red O staining. This assay was performed as previously mentioned [3]. For quantification of lipid content, the oil red O was eluted by adding 100% isopropanol and incubated for 10 minutes before measuring the absorbance or optical density (OD) at 490 nm.

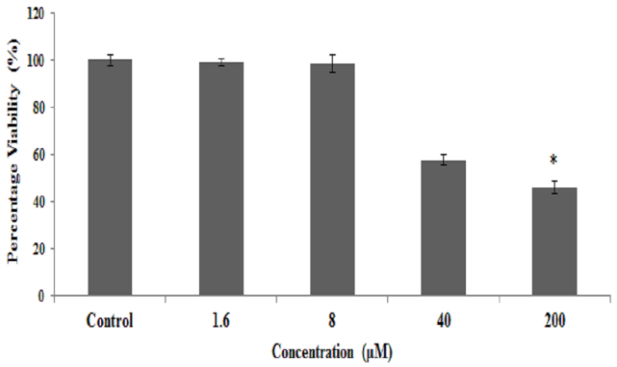
F. *Statistical Analysis*

Values were expressed as means ± SE with three independent experiments. Statistical significance of data was determined using the paired Student's t-test. Values that were less than 0.05 were considered as statistically significant.

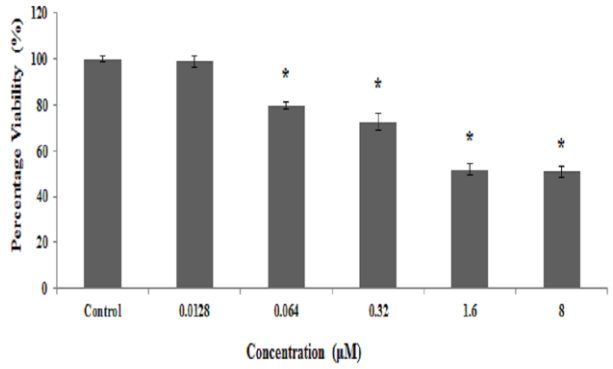
III. Results and Discussions

A. *Effect of Oligomycin and Antimycin A on the Viability of 3T3 -L1 Adipocytes*

The viability assay was used to determine the suitable concentration and possible cytotoxic effects of both drugs on 3T3-L1 adipocytes. It has been observed that oligomycin at the concentration of 200 and 40 µM inhibited the cell viability with the decrease of 46% and 58%, respectively compared to control. The highest concentration of oligomycin that did not decrease cell viability was 8 µM (Fig. 1(a)). Accordingly, this dose was chosen to trigger mitochondrial dysfunction through inhibition of ATP synthase activity on the differentiated 3T3-L1 adipocytes. For antimycin A, it has been found that the cell viability did not decrease at the concentration of 0.0128 µM compared to control, indicating that this dose was suitable to be used for triggering mitochondrial dysfunction through impairment of mitochondrial complex III of ETC (Fig. 1(b)).



(a)



(b)

Figure 1. (a) Cell viability assay on oligomycin treated cell; (b) Cell viability assay on antimycin A treated cell. All values were presented as means ± SD of three independent experiments.

B. *Effects of Oligomycin and Antimycin A on the Cellular ATP Contents of 3T3 -L1 Adipocytes*

The cellular integrity of the mitochondria in the treated and control cells was being assessed through the measurement of the cellular ATP content. The purpose of this analysis was to verify the dysfunctional of intracellular mitochondria ATP contents in the cells for further biological assays.

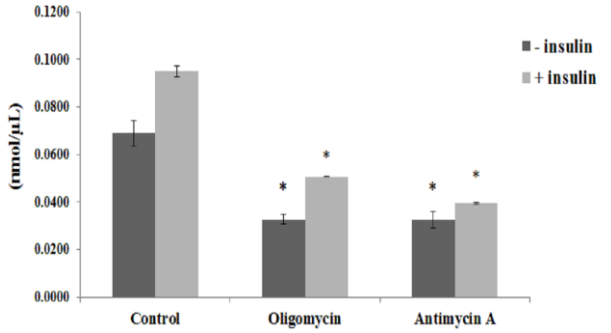


Figure 2. The measurement of intracellular ATP content ration in treated and control cells. Data were presented as the mean ± SD (n=6). * $p<0.05$ compared to the control cells.

The elevations of intracellular lipid accumulations in various peripheral tissues such as skeletal muscle and liver are strongly associated with decreased mitochondrial functions [5], [6]. In order to examine the importance of mitochondrial function under basal (no insulin) and insulin stimulation, cells were treated with mitochondrial inhibitors for 8 hours. Cells treated with the mitochondrial inhibitors presented with the reduced basal and insulin stimulated ATP concentration (Fig. 2). It has been observed that both oligomycin and antimycin A significantly decreased insulin stimulated cellular mitochondrial ATP content within the cell by 47% and 59% while a significant depletion of mitochondrial ATP content by 53% and 54% in the absence of insulin, respectively. The exact mechanisms involved in the insulin resistance induced by mitochondrial dysfunction are not yet fully characterized [2]. Here, the effects of several mitochondrial inhibitors on mitochondrial functions in 3T3-L1 adipocytes were investigated. Mitochondrial ATP generation is so crucial for the majority of the cell functions [7]. ATP is expected to be essential to skeletal muscle, liver and adipose tissues in responding to insulin for the inductions of cellular responses requiring high energy. This chemical energy is also necessary for insulin stimulated glycogen and protein synthesis, as well as for glucose uptake. However, in the events of mitochondrial dysfunctions, insulin cannot be utilized for the energy productions due to induction of various stresses signalling cascade pathways that interrupt numerous cellular pathways in mitochondria.

C. *Effect of Oligomycin and Antimycin A on the Lipolysis Activity of 3T3 -L1 Adipocytes*

To assess the effects oligomycin and antimycin A on the lipolysis activity of 3T3-L1 adipocytes, free fatty acids and glycerol accumulation in the culture media were measured. As shown in the Fig. 3 (a) and (b), the significant dose increased the amount of free fatty acids and glycerol released into the media.

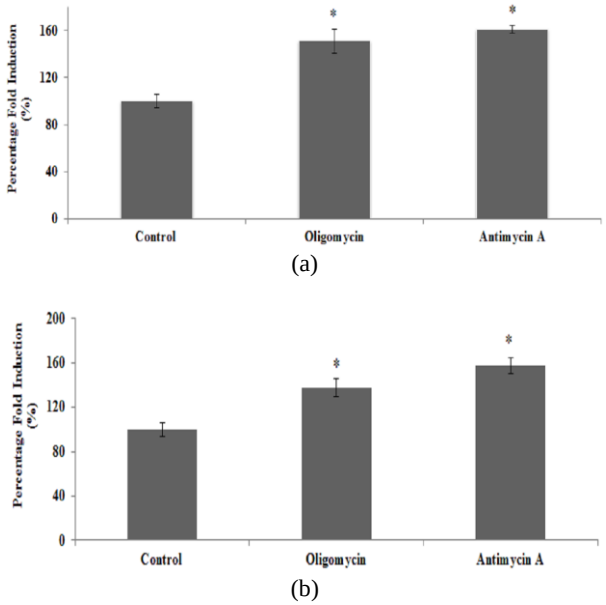


Figure 3. (a) The relative amount of free fatty acids release into the media of both treated and control cells; (b) The relative amount of glycerol release into the media of both treated and control cells. Data were presented as the mean ± SD (n=3). *, $p<0.05$ compared to the control cells.

As compared to non-treated cells (control), oligomycin and antimycin A significantly amplified the relative value of free fatty acids release into the media up to 11% and 10%, respectively for 5 hours treatment (Fig. 3(a)). Furthermore, the significant upsurge in the amount of glycerol released after 5 hours treatments were observed in the treated cell with the relative values of 37% and 57% compared to control (Fig. 3(b)). The excess accumulation of free fatty acids in the media of adipose tissues might be due to impairment of mitochondrial functions that can systematically lead to the increased rate of lipolysis in the body. In the event of increased systemic lipolysis, there is an augmented flow of non-esterified fatty acids (NEFA) into non-adipose tissues such as skeletal muscles and liver that subsequently results in the excessive muscular fat storage, associated with insulin resistance and type 2 diabetes [8]. Prolong exposure of lipid and fatty acids intermediates into numerous target tissues activates deleterious mechanisms of the glucose - fatty acids competition cycle that inherently lead to the mitochondrial dysfunction and insulin resistance [9].

D. *Effects of Oligomycin and Antimycin A on the Intracellular Accumulation of Lipid Droplets of 3T3-L1 Adipocytes*

The formation of numerous cytosolic lipid droplets has been observed in 3T3-L1 adipocytes treated with oligomycin (Fig. 4(d)) and antimycin A (Fig. 4(c)), as visualized using Oil Red O staining. The normal differentiated cells that were treated with adipogenic cocktails (MDI) exhibited a typical adipocyte phenotype with round-shaped lipid droplets. However, the lipid

droplets in both drugs treated cells formed larger and prominent lipid vesicles with scattered and abnormal lipid droplet distributions compared to control cells (Fig. 4(b)).

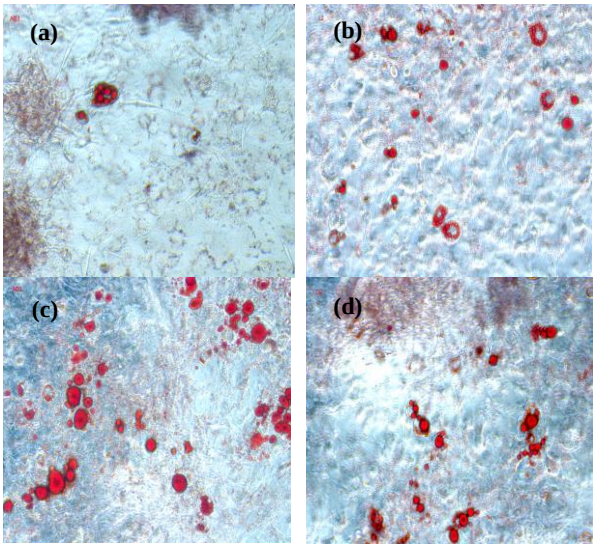


Figure 4. The effect of oligomycin and antimycin A on adipocyte differentiation (a) Untreated (DMSO control), (b) MDI, (c) Antimycin A treated cell and (d) oligomycin treated cell.

As shown by Oil Red Oil elution, treatment with both mitochondrial inhibitors increased intracellular fat accumulation up to 48% and 45% relative to MDI-control treated cell (Fig. 5). In the regulation of insulin resistance, the increases of lipid intermediate are strongly associated with the impairment of mitochondrial functions [10]. The increase of lipid concentration in the specific target tissues may lead to the increase of incomplete fatty acids oxidation and caused the abnormal accumulation of certain lipid intermediates such as ceramides, diacylglycerol and acylcarnitine species [11]. These lipid intermediates exert their deleterious effects in the cytosol via interruption of mitochondrial oxidation capacity that can lead to mitochondrial dysfunctions in adipocytes.

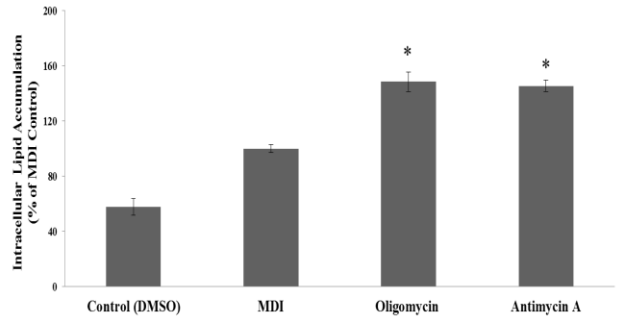


Figure 5. Analysis of intracellular lipid accumulations (OD: 520 nm). All values were presented as means ± SD of three independent experiments. Data were presented as the mean ± SD (n=3). *, $p<0.05$ compared t o t he control cells.

IV. Conclusion

In summary, our result demonstrated that both respiratory inhibitors induced mitochondrial dysfunction via decrease of intracellular ATP concentrations. These metabolic impairments result in the increase of lipolysis activity and an abnormal intracellular lipid accumulation have been observed in the treated cell. Although the direct relationship between mitochondrial dysfunctions and lipolysis requires further investigation, our data provide a mechanistic interaction into the correlation of the impaired mitochondrial functions and other biological processes in the cellular physiological systems.

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