MITOCHONDRIAL DYSFUNCTION OF 3T3-L1 ADIPOCYTES INDUCED BY OLIGOMYCIN AND ANTIMYCIN A

WAN NAJIHAH WAN HASSAN @WAN HUSSEIN

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To my beloved husband, Muhamad Aliemran…

To my parents, brothers and sisters…

To everyone who believed in my abilities….

and supported me through thick and thin….

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ABSTRACT

There are accumulating evidence showing that mitochondrial dysfunction is strongly associated with impaired insulin release and its actions in peripheral tissues. Adipose tissue is one of the important peripheral tissues that regulate the whole-body glucose homeostasis. Metabolic imbalance of energy productions and impaired oxidative phosphorylation in this tissue may lead to mitochondrial dysfunction. The present study sought to investigate the metabolic profile of 3T3-L1 adipocytes in the event of mitochondrial dysfunction. The induction of mitochondria dysfunction in adipocytes were performed by using treatment of oligomycin and antimycin A (AA). Cell viability, triglyceride accumulation, glucose utilization and adenosine triphosphate (ATP) production were analyzed following treatment with these inhibitors. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify the alteration of metabolic profiles associated with mitochondrial dysfunction in adipocytes. The result showed that oligomycin and AA treatment dramatically decreased cell viability by inducing mitochondrial dysfunction at the dose of 8 μM and 0.0128 μM, respectively. The treated cells were found to exhibit increased intracellular accumulation of lipid droplets. Both inhibitors significantly reduced glucose utilization and concomitantly impaired ATP production in adipocytes. Importantly, metabolic profile of adipocytes with mitochondrial dysfunction identified glycerol, arachidonic acid, glutamic acid, ceramide and glycerolphosphoethanolamine (GPE) as predictors for such metabolic perturbations. These findings underscore the potential role of intracellular metabolite in pathogenesis of insulin resistance, thereby suggesting that mitochondrial dysfunction could aid in risk assessment of diabetes.

ABSTRAK

Bukti-bukti kajian menunjukkan bahawa disfungsi mitokondria adalah sangat berkaitan dengan kerosakan pelepasan dan tindakan insulin pada tisu periferal. Tisu adipos adalah salah satu tisu periferal penting yang mengawal homeostasis glukosa seluruh badan. Ketidakseimbangan metabolik dari segi pengeluaran tenaga dan pemfosforilan oksidatif pada tisu sasaran ini boleh membawa kepada disfungsi mitokondria. Kajian ini bertujuan untuk mengenalpasti profil metabolik sel lemak 3T3-L1 sekiranya berlaku kegagalan fungsi mitokondria seiring dengan perubahan yang berlaku di peringkat metabolik. Disfungsi mitokondria dalam *adipocytes* telah diaruh dengan menggunakan oligomisin dan antimisin A (AA). Kebolehidupan sel, pengumpulan trigliserid, penggunaan glukosa dan pengeluaran adenosina trifosfat (ATP) telah dianalisis dengan kehadiran kedua aruhan ini. Kromatografi cecair dengan spektrometri jisim penerbangan (LC-MS/MS) telah digunakan untuk mengenal pasti perubahan metabolik yang dikaitkan dengan disfungsi mitokondria dalam sel lemak. Keputusan menunjukkan bahawa rawatan oligomisin dan AA telah mengurangkan kebolehidupan sel yang mendorong kepada disfungsi mitokondria masing-masing pada dos 8 μM dan 0.0128 μM. Sel sel yang dirawat didapati telah mempamerkan peningkatan pengumpulan titisan lipid dalam sel. Kedua-dua perencat telah mengurangkan penggunaan glukosa dan menjejaskan pengeluaran ATP dalam sel lemak. Perubahan metabolik sel akibat disfungsi mitokondria telah dikenal pasti seperti gliserol, asid arakidonik, asid glutamik, seramida dan gliserolfosfoetanolamina (GPE) sebagai ramalan terhadap gangguan metabolik. Penemuan ini menekankan peranan potensi metabolik intrasel dalam patogenesis rintangan insulin, dan menunjukkan bahawa disfungsi mitokondria boleh membantu dalam penilaian risiko diabetes.

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LIST OF ABBREVIATIONS

AA	-	Antimycin A
ADP	-	Adenosine Triphosphate
AMP	-	Adenosine Monophosphate Activated Protein
ANOVA	-	Analysis of Variance
ASP	-	Acylation stimulating protein
AT II	-	Angiotensin II
ATP	-	Adenosine 5'-Triphosphate
BAT	-	Brown Adipose Tissue
CDC	-	Centers for Disease Control
CETP	-	Cholesteryl ester Transfer Lipase
C/EBP	-	CCAT/Enhancer-Binding Protein
CoA	-	Coenzyme A
CREB	-	cAMP responsive element-binding protein
CRP	-	C-reactive protein
DMEM	-	Dulbecco'c Modified Eagle's Medium
DMSO	-	Dimethyl Sulfoxide
ECM	-	Extracellular Matrix
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme-linked immunosorbent assay
EPAs	-	esterified fatty acids
ETC	-	Electron Transport Chain
FAS	-	Fatty acid synthase
FAT-1	-	Fatty acid transport protein 1
FBS	-	Fetal Bovine Serum
FCS	-	Fetal Calf serum
FFA	-	Free Fatty Acid

FM	-	Freeze Medium
GC	-	Gas chromatography
GLUT 4	-	Glucose transport type 4
GPE	-	glycerophosphophotoethanolamine
HDL	-	high density lipoprotein
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	-	High-Performance Liquid Chromatography
Hnf	-	hepatocyte nuclear factor
HSL	-	Hormone Sensitive Lipase
IBD	-	Institute Bioproduct Development
IBMX	-	isobutyl-1-metylxanthine
IDF	-	International Diabetes Federation
IGF-1	-	insulin like growth factor 1
IL-6	-	Interleukin 6
IMCL	-	Intramyocellular Lipid
IMS	-	Intermembrane Space
INM	-	Inner Membrane
IRS-1	-	Insulin Receptor Substrate-1
LC	-	Liquid Chromatography
LDL	-	low density lipoprotein
LPL	-	Lipoprotein Lipase
Lyso-PC	-	Lyso-phosphatidylcholines
MCP-1	-	Macrophage chemo attractant protein-1
MPP	-	Mass profiler professional
MS	-	Mass Spectrometry
MW	-	Molecular Weight
NADH	-	Nicotinamide Adenine Dinucleotide
NCBs	-	Newborn calf serum
NEFA	-	non esterified fatty acids
NMR	-	Nuclear Magnetic Resonance
OD	-	Optical Density
OMM	-	Outer Membrane
OXPHOS	-	Oxidative Phosphorylation

OxPs	-	oxidized phospholipids
PAI-1	-	Plasminogen activator inhibitor-1
PBS	-	Phosphate Buffer Saline
PCDL	-	personal compare database and library
PCX	-	pyruvate carboxylase
PGC-1	-	Peroxisome Proliferator Activated Receptor Coactivator-1
PHBs	-	prohibitins
PI-3	-	Phosphatidylinositol-3
PPAR	-	Peroxisome Proliferator Activated Receptor
PTP	-	permeability transition pore
QC	-	Quality Control
Q-TOF	-	Quaruple time of flight
ROS	-	Reactive Oxygen Species
SD	-	standard deviation
SNPs	-	Single-Nucleotide Polymorphisms
SOD	-	superoxide dismutase
TCA	-	Tricarboxylic Acid
TG	-	triglyceride
T2DM	-	Type 2 Diabetes Mellitus
TNF	-	Tumor Necrosis Factor
UCP-1	-	Uncoupling Protein-1
UPLC	-	Ultra Performance Liquid Chromatography
USA	-	United States of America
WAT	-	White Adipose Tissue
Zfp	-	Zinc finger protein

LIST OF SYMBOLS

C	-	Celcius
^{13}C	-	carbon 13
cm^2	-	centimeter square
g	-	gram
h	-	hour
^{1}H	-	hydrogen
H_2O	-	water
L	-	litre
min	-	minute
Mm	-	Millimeter
nm	-	nanometer
NaOH	-	sodium hydoxide
O_2	-	oxygen
^{31}P	-	phosphorus
U	-	enzyme Unit
~	-	approximately
<	-	less than
°	-	degree
%	-	percent
α	-	alpha
β	-	beta
γ	-	gamma
μ	-	micro

CHAPTER 1

INTRODUCTION

1.1 Background of study

The role of mitochondria in adipocyte metabolism, specifically on mitochondrial respiratory function has been emphasized in recent studies (Bakar *et al*., 2014). Indeed, mitochondrial abnormalities was known to lead to several pathological conditions characterized by aberrant insulin signaling, abnormal glucose utilization, lipid metabolism disorder and excessive triglyceride (TG) accumulation in several cell types. Interestingly, mitochondrial dysfunction in adipocyte was associated with type 2 diabetes mellitus (T2DM), resulting from elevated free fatty acid (FFA) levels and impaired oxidative phosphorylation (OXPHOS).capacity (Guilherme *et al*., 2008; Muoio and Neufer, 2012).

Diabetes is characterized by high blood sugar level due to systemic malfunction of glucose regulation. There are 3 types of diabetes. Type 1 diabetes is as a result of autoimmune destruction of the insulin-producing beta cells in the pancreas. A sufferer with this type of diabetes requires exogenous insulin for survival in order to avoid ketoacidonesis. Type 2 diabetes is characterized by insulin resistance and has been linked with metabolic syndrome and a doubled risk for

cardiovascular disease. Meanwhile, gestational diabetes may occur during pregnancy. Among of these types, type 2 diabetes is the most prevalence form of diabetes characterized by decreased insulin sensitivity in various target tissues. International Diabetes Federation (IDF) estimated 366 million people worldwide suffered from this disease in 2011, and projected to rise to 552 million in 2030 (Whiting *et al*., 2011). A large proportion of these individuals remain undiagnosed due to lack of symptoms early on the disease. Until now, there is no definitive clinical intervention to reverse diabetes and the understanding of its disease mechanism is still an open discussion.

Several studies have postulated that prominent feature of type 2 diabetes are regarded as mitochondrial dysfunction (Lowell and Shulman., 2005; Martin *et al*., 2014). Disruptions of mitochondrial function are known to cause an insulin-deficient form of diabetes. Liu *et al*. (2012) demonstrated that reduction of adipogenic markers, typically prohibitins (PHBs) as a result of impairment of mitochondrial activity. The impairment of PHB cause reduction of mitochondrial content, impairment of mitochondria complex 1 activity and excessive production of reactive oxygen species (ROS) that may lead to metabolic disorder.

Recently, the use of the 3T3-L1 adipocytes as differentiation model to study their role in metabolic syndrome were increasing. The adipocyte is a lipid depository sites and a key metabolic regulator responsible for the production of cytokines, metabolic substrate and adipokines which influence the whole body metabolic activity (Roberts *et al* , 2009) . Therefore, numerous basic and clinical researches have been devoted toward the dysregulation of metabolism within adipose tissue that may contribute to the progressions of T2DM, metabolic syndrome and obesity.

Even though there are several *in vitro* studies performed using 3T3-L1 adipocyte in analyzing the mechanistic of insulin resistance, glucose uptake and lipid metabolism cell culture models that highlighted the metabolite profile in adipocyte dysfunction of T2DM is still lacking. Determination of metabolite concentrations

and detections provide complementary information to the mechanistic understanding of a particular diseases. The methods based on Mass Spectrometry (MS) remain dominant analytical platforms. Mass spectrometry-based metabolomics provide a quantitative analysis with high selectivity and sensitivity. These approaches also have potential to identify metabolite of interest and its aetiological pathways for biological interpretations.

1.2 Problem Statement

Over a past few years, adipose tissue has been acknowledge to exert the whole blood glucose homeostasis and modulate various metabolic functions in both normal and diseases states such as insulin resistance, T2DM, hypertension, dyslipidemia and even some cancers (De Pauw *et al*., 2009; Sam and Mazzone, 2014). Recently, type 2 diabetes contributes to major epidemic cases in developing nations especially among young people (Shaw *et al*., 2010; Weill *et al*, 2012).

Adipocyte cell store and release triglycerides (TGs) as well as regulate several metabolic functions in endocrine activity since adipocyte secretes a specific hormone and proinflammotary cytokines, namely, adipokines or adipocytokines which provide an extensive network of communication within adipose tissue and other organ. Importantly, this network of communication regulate energy homeostasis in organism (Ahima, 2006). However, the interaction adipose tissue depots are distinguished between white adipose tissue (WAT) and brown adipose tissue (BAT). Although BAT originates from the myogenic lineage, it shares many features of WAT (Seale *et al*., 2008). WAT has been recognized as main storage of excess energy from food intake as metabolic active, lipid storage and endocrine organ. A better understanding mechanism involved in adipocyte differentiation is required to reveal mechanism underlying diabetes and insulin resistance. This

understanding is important to develop new therapeutic approach that directly target intracellular pathway in adipocytes.

Although adipocyte dysfunction linking obesity to insulin resistance have been recently reviewed (Guilherme *et al*., 2008), the role of mitochondrial dysfunction associated with T2DM in and mature adipocyte differentiation had hardly been addressed. Moreover, the metabolic activities are different between subcutaneous and visceral WAT, depending on their anatomical position and mitochondrial content (Wajchenberg, 2000). Deveaud *et al*. (2004) reported that, in the visceral depot, adipocytes are richer in mitochondrial compared to subcutaneous adipocytes. Mitochondrial is known to play an essential role in physiological and metabolic activity in the pathology of human disease including type 2 diabetes.

Although there is clear association between insulin resistance, type 2 diabetes (T2DM) and mitochondrial dysfunction, there is no compelling evidence of metabolite isolation as potential marker in the development of diabetes. Thus, the aim of this study has been directed towards elucidation of the cellular metabolite profile of mitochondrial dysfunction induced diabetic cell cultured that involved in the development insulin resistance and type 2 diabetes.

In the study of mitochondria dysfunction in the adipose tissue, we hope to elucidate the impact of mitochondrial activity in adipocyte biology. These ideas may lead to the novel understanding of the effects of mitochondrial stress on adipocytes and their systemic metabolic functions as well the potential metabolite profile as a biomarker panel that contribute to dysfunctional mitochondrial in a diabetes state.

1.3 Research Hypothesis

Metabolite profiling of mitochondria dysfunction activity detectable in cultured cells is a robust biomarkers for insulin resistance/type 2 diabetes.

1.4 Objective of the Research

In this research, the metabolite profiling of mitochondria dysfunction in 3T3-L1 adipocyte cell was investigated. Drug-induced mitochondrial activity in 3T3-L1 adipcoytes utilizing various concentrations may affect the mitochondrial biogenesis and the non-targeted approach of metabolomics were studied. The objective of this research was:

To establish the metabolomics marker in oligomycin and Antimycin A (AA)-induced mitochondria dysfunction in 3T3-L1 adipocyte.

1.5 Scope of the Research

In order to achieve the objectives of the research, the experimental work was divided into three major scopes. The scopes of the research were:

i) Preparation of dysfunctional mitochondria 3T3-L1 adipocytes model using oligomycin and Antimycin A.

ii) Analysis of the treated cells of oligomycin and antimycin A by determining cytotoxicity effect, lipid content, glucose utilization, and ATP production.

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