

CELLULAR FUNCTIONAL ROLES OF CELASTROL ON MITOCHONDRIAL
DYSFUNCTION-INDUCED INSULIN RESISTANCE

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Special dedication to my beloved

Father

Abu Bakar Bin Osman

Mother

Faezah Binti Idris

Siblings

Nor Shafinaz Binti Abu Bakar

Siti Mayuha Binti Abu Bakar

Maisarah Binti Abu Bakar

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Thanks for all the tremendous love, understanding, prayer, advices and motivations during the hard times.

“My Lord! Increase me in knowledge”

Quran 20:114

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ABSTRACT

There are compelling evidence showing that mitochondrial dysfunction and low-grade chronic inflammation in several peripheral tissues may attribute to the central pathophysiological mechanism of insulin resistance and type 2 diabetes. Celastrol, a pentacyclic-triterpene, is an established anti-inflammatory agent from the root of *Tripterygium wilfordii* that has been used for centuries as medicament to treat numerous inflammatory diseases. As its therapeutic treatment is increasingly being recognized, the present study sought to investigate the functional roles of celastrol upon mitochondrial dysfunction and insulin resistance induced by mitochondrial respiratory inhibitors in insulin responsive cells. The glucose uptake activity, mitochondrial functions, lipolysis, intracellular lipid accumulation and a number of signaling pathways were investigated using cell-based assays and western blot analyses. The optimum doses of celastrol in improving insulin-stimulated glucose uptake of mitochondrial inhibitors-treated 3T3-L1 adipocytes, human skeletal muscle and C3A human liver cells were 5, 15 and 30 nM, respectively. Celastrol treatment for 48 hours improved the mitochondrial activities and decreased the mitochondrial superoxide productions. The integrity of mitochondrial dynamics was restored via substantial changes in mitochondrial fusion and fission. Furthermore, celastrol prevented the amplified level of cellular oxidative damages where the production of pro-inflammatory cytokines in cultured cells was greatly down-regulated. The release of free fatty acids and glycerol from conditioned media of adipocytes and hepatocytes were reduced after celastrol treatment. The relative amount of intracellular lipid accumulation was decreased in celastrol-treated cells with mitochondrial dysfunction. Importantly, celastrol enhanced the phosphorylation of amino acid residues of insulin receptor substrate 1 (IRS1), serine/threonine kinase (Akt/PKB) and Akt substrate 160 (AS160) proteins in insulin signaling pathways with amplified expression of 5' adenosine monophosphate-activated protein kinase (AMPK) protein in human myotubes and hepatocytes. The metabolic effects of celastrol were also accompanied with the attenuation of nuclear factor-kappa B (NF- κ B) and diminished activation of the protein kinase C (PKC) isoforms in insulin resistant cells. The protein expression of glucose transporter 4 (GLUT4) was normalized by celastrol in adipocytes and human myotubes while reduced GLUT2 protein expression was observed in hepatocytes, signifying its ameliorative properties in enhancing insulin sensitivity of these *in vitro* disease models. Collectively, these results unequivocally suggested that celastrol may be advocated for use as a potential therapeutic molecule to protect against mitochondrial dysfunction and inflammation in the development of insulin resistance and type 2 diabetes.

ABSTRAK

Terdapat banyak bukti menunjukkan bahawa ketidakfungsian mitokondria dan keradangan kronik tahap rendah dalam beberapa tisu periferel telah dikaitkan dengan mekanisme patofisiologikal pusat dalam kerintangan insulin dan penyakit diabetes jenis 2. *Celastral*, sejenis pentasiklik-triterpena, merupakan agen anti-radang daripada akar kayu pokok *Tripterygium wilfordii* yang telah sekian lama digunakan sebagai ubat untuk merawat pelbagai penyakit radang. Memandangkan kepentingan rawatan terapeutik kini semakin disedari, kajian ini bertujuan untuk mengkaji peranan fungsi *celastral* ke atas ketidakfungsian mitokondria dan kerintangan insulin yang disebabkan oleh perencat respirasi mitokondria dalam sel-sel yang responsif terhadap insulin. Aktiviti pengambilan glukosa, fungsi-fungsi mitokondria, lipolisis, pengumpulan lipid intraselular dan beberapa laluan isyarat telah dikaji menggunakan beberapa cerakinan berasaskan sel dan analisis pemendapan *Western*. Dos *celastral* yang optimum dalam meningkatkan pengambilan glukosa yang diransangkan oleh insulin dalam sel adiposit 3T3-L1, otot rangka manusia dan sel hati manusia C3A yang dirawat dengan perencat mitokondria ialah masing-masing 5, 15 dan 30 nM. Rawatan *celastral* selama 48 jam meningkatkan aktiviti mitokondria dan mengurangkan pengeluaran radikal superoksida mitokondria. Integriti dinamik mitokondria telah dipulihkan melalui perubahan besar dalam gabungan dan pembelahan mitokondria. Tambahan pula, *celastral* menghalang kerosakan sel oksidatif yang mana pengeluaran sitokin pro-radang dalam sel-sel dikurangkan. Pelepasan asid lemak bebas dan gliserol daripada media sel adiposit dan hepatosit telah dikurangkan selepas rawatan *celastral*. Jumlah relatif pengumpulan lipid intraselular telah menurun dalam ketidakfungsian mitokondria sel yang dirawat dengan *celastral*. *Celastral* meningkatkan pemfosforilan beberapa jujukan asid amino daripada protein-protein substrat reseptor insulin (IRS1), serina/treonina kinase (Akt/PKB) dan substrat Akt 160 (AS160) dalam laluan isyarat insulin dengan ungkapan protein 5' adenosina monofosfat-diaktifkan kinase (AMPK) ditingkat dalam miotiub dan hepatosit manusia. Kesan metabolik *celastral* juga disertakan dengan pengurangan faktor nuklear kappa B (NF- κ B) dan protein kinase C (PKC) dalam sel kerintangan insulin. Ungkapan protein glukosa pengangkut 4 (GLUT4) telah dinormalkan oleh *celastral* dalam sel adiposit dan miotiub manusia manakala pengurangan ungkapan protein glukosa pengangkut 2 (GLUT2) diperhatikan dalam hepatosit, lantas memperlihatkan sifatnya dalam memperbaiki sensitiviti terhadap insulin dalam sel-sel model penyakit ini. Secara keseluruhannya, keputusan ini menunjukkan bahawa *celastral* berpotensi untuk digunakan sebagai molekul terapeutik bagi melindungi daripada ketidakfungsian mitokondria dan keradangan dalam kerintangan insulin dan penyakit diabetes jenis 2.

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LIST OF ABBREVIATIONS

8-OHdG	-	8-hydroxydeoxyguanosine
ADP	-	adenosine diphosphate
AMA	-	antimycin A
AMPK	-	adenosine monophosphate-activated protein kinase
ATP	-	adenosine triphosphate
BSA	-	bovine serum albumin
CaCl ₂	-	calcium chloride
CO ₂	-	carbon dioxide
CoA	-	coenzyme A
CPT-1	-	carnitine palmitoyltransferase 1
DAG	-	diacylglycerols
DCFDA	-	2',7' –dichlorofluorescein diacetate
DMEM	-	Dulbecco's modified eagle's medium
DNA	-	deoxyribonucleic acid
ETC	-	electron transport chain
FADH ₂	-	flavin adenine dinucleotide
FBS	-	fetal bovine serum
GLUT	-	glucose transporter
H ₂ O	-	water
H ₂ O ₂	-	hydrogen peroxide
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSF1	-	heat shock factor protein 1
HSP	-	heat shock protein
IKK	-	IκB kinase

IL-1 β	-	interlekin-1 β
IL-6	-	interleukin-6
IRS	-	insulin receptor substrate
I κ B α	-	inhibitor of kappa B
KH ₂ PO ₄	-	monopotassium phosphate
MDA	-	malondialdehyde
MgSO ₄	-	magnesium sulfate
mRNA	-	messenger ribonucleic acid
MRS	-	magnetic resonance spectroscopy
mtDNA	-	mitochondrial DNA
MTT	-	3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium
NADH	-	nicotinamide adenine dinucleotide
NaOH	-	sodium hydroxide
NF- κ B	-	nuclear factor-kappa B
O ₂ -•	-	superoxide radical
OD	-	optical density
PBS	-	phosphate buffer saline
PI3K	-	phosphatidylinositol-3-kinase
PKC	-	protein kinase C
PPAR γ	-	peroxisome proliferator-activated receptor gamma
ROS	-	reactive oxygen species
TCA	-	tricarboxylic acid
TNF- α	-	tumor necrosis factor- α
v/v	-	volume per volume
w/v	-	weight per volume

LIST OF SYMBOLS

%	-	percent
μg	-	microgram
cm	-	centimeter
g	-	gram
g	-	relative centrifugal force
g/mol	-	gram per mol
h	-	hour
L	-	liter
mg/L	-	milligram per liter
min	-	minutes
mL	-	milliliter
mm	-	millimeter
nM	-	nano molar
$^{\circ}\text{C}$	-	degree Celsius
rpm	-	revolution per minute
t	-	time
α	-	alpha
β	-	beta
γ	-	gamma
δ	-	delta
$\Delta\Psi\text{m}$	-	mitochondrial membrane potential
θ	-	theta
κ	-	kappa
μL	-	microliter

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Type 2 diabetes mellitus is a devastating metabolic disorder characterized by insulin resistance and linked to various metabolic syndromes such as hormonal imbalance, hypertension, hyperglycemia and excess fatty acids in blood circulation [1]. The biological determinant such as genetic factors is involved in the pathogenesis of type 2 diabetes [2,3]. One of the first-degree relatives who had family history suffered from type 2 diabetes is conferred to have been three-fold increased risk of developing the disease [3–5]. On the other side of the scale, during the last few decades, the dramatic increases in incidence and prevalence rates of this disorder are intimately observed in developed and developing countries [6]. Undoubtedly, it is becoming increasingly difficult to ignore the influence of environmental factors in the onset of such disease. It can be signified that the concerted actions of both genetic and environmental factors such as malnutrition, psychological stresses, smoking, alcohol intake, aging and sedentary lifestyles are considerably linked together towards the development of type 2 diabetes and its co-morbidities [7].

In the following years, the roles of mitochondrial dysfunction-induced inflammation towards progression of insulin resistance, the forerunner of type 2 diabetes mellitus, have acquired important new dimensions [8–10]. Indeed, a

multitude of studies have discovered that the impairments of mitochondrial functions in skeletal muscles, liver and adipose tissues of both human and animal diseased subjects are etiologically associated with low-grade chronic inflammation [11,12]. In light of data indicating a pathophysiologic role of mitochondrial dysfunction in the occurrence of inflammation and insulin resistance, it is intriguing to hypothesize that the metabolic adaptations observed in these target tissues may affect the whole-body metabolism as a whole. To a smaller extent, it is now becoming clear that the derangements of cellular inflammatory mediators are inextricably linked to oxidative stress and reduced mitochondrial functions in insulin resistance state [9]. Although the molecular details of such signaling remain enigmatic, extensive data advocated that several destructive activators can lead to the intense oxidation of mitochondrial DNA, lipid and protein, resulting in the advancement of pro-inflammatory cytokines production via activation of nuclear factor-kappa B (NF- κ B) signaling pathways in a number of metabolic tissues [8,11,13]. Thus, further therapeutic research targeting these regulatory pathways and its ameliorative mechanisms in these peripheral tissues may provide an insight towards effective treatments of such disorders.

The concerted understanding of the pathogenesis of type 2 diabetes and insulin resistance persists to drive personalized approaches to treatment with the minimized side effects. Aside from new synthesized drugs, the search for more effective and safe anti-diabetic agents continues to be an area of research interest to expand the therapeutic armamentarium. The use of active compounds derived from plants for use as drugs and medicines in alleviating various metabolic diseases is attracting increasing attention. Celastrol is an established active ingredient of natural quinone methide triterpenoid isolated from plant family *Celastraceae* (*Tripterygium wilfordii* Hook F.), the traditional Chinese medicine called “Thunder of God Vine”. This compound exhibits a number of biological activities including anti-oxidant, anti-inflammatory and anti-cancer properties [14]. The mechanistic actions of celastrol on the cellular targets are poorly understood, thereby impeding its application in clinical studies. Though, mounting evidences documented that celastrol has its own unique capability to inhibit NF- κ B transcription factors and its downstream targets in various cell types without affecting DNA-binding activity of activator protein 1 (AP-1) [15–17]. Numerous studies to define its pharmacological mechanism showed that it

suppresses many steps of oxidative stress induction via NF- κ B inhibition and modulates several inflammatory responses in peripheral tissues. Hence, subsequent experimental approaches in evaluating the attributive roles of this compound in hindering the activation of inflammatory pathways relative to mitochondrial functions and insulin signaling activities in metabolic diseases are of great interest [18]. On the basis of recent evidence, the search for more effective and safer natural anti-inflammatory agents with multiple ameliorative properties in enhancing insulin sensitivity should be recognized to be an important area of investigation.

1.2 Problem Statement

Mitochondria have a plethora of physiological and pathological functions in several signaling pathways including regulation of calcium (Ca^{2+}) homeostasis, orchestration of apoptosis, and mitochondrial superoxide production [19]. Presumably through its ability to regulate innumerable biological functions, any perturbation in these central processes may greatly alter the cellular and systemic functions of the organisms with dire consequences. Correspondingly, the multitude of studies revealed that the specific perturbations of mitochondrial oxidative phosphorylation including changes in mRNA levels of mitochondrial markers, enzymatic activities and substrate oxidation are allied to the progression of insulin resistance, hepatic steatosis and type 2 diabetes [9,20–22]. Among these, it is now acceptable that the reduced oxidations of several important fuels such as glucose and fatty acids can exacerbate the disease along with impaired oxidative metabolism.

Accumulating evidence suggests that skeletal muscle, liver and adipose tissues are among the primary target tissues for various metabolic activities relative to cellular mitochondrial energy homeostasis and functions [9]. Functional disturbances in these tissues can, therefore, theoretically contribute to several metabolic impairments. The substantial evidence from previous literatures pointed out that the impaired activity of Complex I and III in the mitochondrial electron transport chain and reduced adenosine

triphosphate (ATP) synthase proteins are major contributors to oxidative stress in rat fatty liver and diabetic patients [22–24]. These tissues are significantly affected in the progression of insulin resistance and type 2 diabetes, advocating that these tissues can be one of the promising targets for development of new diabetes drugs [25]. It is also important to note that current modern therapies in this field are extensively engaged towards the development of new therapeutic intervention of the disease rather than prevention. The exploration of new preventive strategies involving food and drink-containing bioactive compounds remains a priority in order to mitigate the severity of such disease progression.

In recent years, emerging evidence has been gathered to support the notion that an increase of oxidative stress, mitochondrial damage and exacerbated inflammation are among the key features of obesity and type 2 diabetes [8,10]. The concerted actions of both acute and chronic inflammation with augmented superoxide free radicals productions can lead to further reduce the ATP generation, consequently impeding insulin signaling activities in some peripheral tissues. In that sense, activations of redox-sensitive inflammatory pathways via NF- κ B and c-Jun N-terminal kinase (JNK) signaling by mitochondrial dysfunction have been postulated as an adaptive system of cellular stresses towards overwhelmed generation of reactive oxygen species (ROS) [26]. To a lesser extent, the chronic stimulation of these inflammatory pathways have been recognized as the “main culprits” that contribute to the progression of type 2 diabetes. Still, the precise mechanisms linking inflammation and mitochondrial dysfunction in metabolic tissues are still rather ambiguous. Although it is broadly appreciated that oxidative stress and inflammation lead to development of insulin resistance, the therapeutic interventions in modulating these mitochondrial dysfunction-induced inflammations that lead to insulin resistance are relatively scarce. Hence, further therapeutic strategy and prevention should be modulated towards inhibition of these detrimental pathways while boosting the metabolic pathways that promote enhanced cellular bioenergetics.

In the search for novel treatments, the present study was designed to establish the *in-vitro* disease model of mitochondrial dysfunction-mediated insulin resistance

and inflammation in insulin responsive cells using mitochondrial inhibitors. As mitochondrial dysfunction is strongly associated with the activation of NF- κ B inflammatory signaling pathways in these disease models, the therapeutic treatment in modulating these pathways is imperatively needed. The use of celastrol in ameliorating such metabolic impairments related to mitochondrial dysfunction and inflammation in these *in-vitro* disease models was undertaken.

1.3 Objective

The central objective of this study was to investigate the functional roles of celastrol upon mitochondrial dysfunction-induced insulin resistance in insulin responsive cells.

1.4 Scopes of the Study

In order to achieve this objective, three research scopes were carried out:

1. To establish the *in vitro* disease models of mitochondrial dysfunction-induced insulin resistance in 3T3-L1 adipocytes, human skeletal muscle and C3A human liver cells.
2. To evaluate the attributive roles of celastrol in modulating glucose uptake, inflammatory signaling, mitochondrial functions, lipolysis and intracellular lipid accumulation in these mitochondrial inhibitor-treated cells.
3. To explore the metabolic effects of celastrol on the phosphorylation sites of insulin signaling pathways, AMP-activated protein kinase (AMPK), protein kinase C (PKC) isoform activations and glucose transporters protein expression in the *in vitro* disease models.

1.5 Significances and Original Contributions of the Study

This investigation offers several contributions in the area of preventive and personalized medicine in treating mitochondrial dysfunction associated with insulin resistance and type 2 diabetes. The contributions are as follows:

- i. To the best of current knowledge, this study is one of the first reports towards specific establishment of the *in vitro* disease models for mitochondrial dysfunction-induced insulin resistance in insulin responsive cells. Currently, a number of studies in these areas are mainly focused using high level of glucose and free fatty acids in the media to induce insulin resistance in the cells. However, increasing evidence shows that the onsets of mitochondrial dysfunction, oxidative stress and peripheral insulin resistance in human and animal disease models are mainly triggered by the impaired mitochondrial respiratory chain activity (complex I and III) and reduced ATP-oxidative phosphorylation. Thus, there are compelling reasons to establish the *in vitro* disease models through specific inhibition of mitochondrial respiratory chain activity and ATP synthase to mediate insulin resistance in the cells in order to unravel the exact metabolic associations between insulin resistance and impaired oxidative metabolism.
- ii. Although mitochondrial dysfunction is strongly associated with inflammation, the roles of several key intracellular signaling cascades in regulating mitochondrial functions have not been fully characterized. Therefore, an exploration of *in vitro* functional roles of celastrol, an anti-inflammatory compound, in the event of mitochondrial dysfunction-induced insulin resistance may provide beneficial insight on the novel understanding of the therapeutic intervention and cellular mechanisms underlying deteriorated mitochondrial functions, inflammation and insulin resistance.

- iii. Celastrol has been reported to possess a potent anti-oxidant, anti-inflammation and anti-cancer in a number of disease models. New emerging *in vivo* data suggest that celastrol exercises its beneficial properties through amelioration of insulin resistance, weight gain and attenuation of numerous detrimental occasions in animal models. In contrast to its emerging role in various animal models of such diseases, there is a paucity of information regarding the *in vitro* effects of celastrol on insulin sensitivity and no comparative studies that relate to the use of celastrol in treating inflammation with reduced mitochondrial functions in the disease settings. To date, the specific studies on the mechanistic actions of celastrol in the peripheral tissues relative to mitochondrial dysfunction and insulin resistance have not been verified, hindering the current status of celastrol usage at the clinical trials. Thus, there seems to be great potential of further therapeutic intervention to study these mechanistic actions. To this end, the present study contributes to the new findings on the use of celastrol against the development of mitochondrial dysfunction and insulin resistance.

1.6 Thesis Structure and Organization

This thesis is divided into five chapters. Chapter 1 covers a brief overview of the research backgrounds, problems statement, central objective, scopes of analyses, originality and significant contributions of the study.

Chapter 2 offers an overview of type 2 diabetes, insulin resistance and inflammation with the inclusion of the roles of mitochondrial dysfunction and NF- κ B signaling pathways in the settings of such disorders. The literature also highlight the

current mechanistic roles of celastrol in the development of various metabolic diseases.

Chapter 3 covers the overall methodologies used for the cell-based assays in investigating and evaluating the attributive roles of celastrol on mitochondrial dysfunction-induced insulin resistance in 3T3-L1 adipocytes, human skeletal muscle and C3A human liver cells.

Chapter 4 presents the comprehensive results and discussions on the ameliorative properties of celastrol treatment on glucose uptake activity, mitochondrial functions, lipolysis, lipid distribution, pro-inflammatory cytokines release, intracellular insulin signaling pathways and its downstream target proteins in these *in vitro* disease models. The general proposed mechanisms of celastrol in 3T3-L1 adipocytes, human skeletal muscle and C3A human liver cells were also presented.

Chapter 5 provides the overall summary of the research findings and specific future recommendations for the upcoming works.

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