A STUDY ON THE EFFECTS OF INSULIN, TNF-ALPHA AND ANTIOXIDANTS ON THE EXPRESSION OF ATP-BINDING CASSETTE TRANSPORTER 1 (ABCA1) IN HEPG2 CELLS

By

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ABSTRACT

A STUDY ON THE EFFECTS OF INSULIN, TNF-ALPHA AND ANTIOXIDANTS ON THE EXPRESSION OF ATP-BINDING CASSETTE TRANSPORTER 1 (ABCA1) IN HEPG2 CELLS

HIA YI LIN

ATP-binding cassette transporter subfamily A member 1 (ABCA1) acts as a transmembrane lipid transporter and it belongs to ABCA subfamily. ABCA1 was proven as a rate-limiting factor in high density lipoprotein (HDL) formation. Moreover, ABCA1 is being correlated with atherosclerosis inversely and hence ABCA1 is an essential therapeutic target in treating atherosclerosis. HepG2 cells were used as a model system in this entire study because ABCA1 was found to be expressed in this cell line. This study was aimed to investigate the effects of TNF-α, insulin and antioxidants on ABCA1 expression in HepG2 cells using quantitative RT-PCR. Prior to qRT-PCR, the cells were treated with different concentrations of insulin. Here, insulin alone was able to suppress ABCA1 mRNA expression in a dose-dependent manner. ABCA1 gene expression was downregulated to 0.46-fold, 0.33-fold, 0.34-fold, 0.35-fold and 0.26-fold with the presence of 1 nM, 5 nM, 10 nM, 50 nM and 100 nM of insulin respectively. A significant ABCA1 reduction to 0.33-fold was observed in 5 nM of insulin treatment. Therefore, 5 nM of insulin was chosen as optimal concentration and this dosage was used in all subsequent treatments. Similar suppressive effect was obtained with the addition of 20 ng/ml TNF-α and 5 nM of insulin. However, in

the presence of antioxidants, the inhibitory effects of TNF-α and insulin were abolished and ABCA1 gene expression was upregulated. Lauric acid and resveratrol augmented the normalised fold expression of ABCA1 gene significantly in a dose-dependent manner in a 24-hour incubation period. In TNF-α-insulin treated HepG2 cells, ABCA1 gene expression was elevated to 1.05-fold, 1.22-fold and 1.72-fold in 5 μM, 10 μM and 20 μM of lauric acid, respectively. With cells post-treated 5 μM, 10 μM and 20 μM of resveratrol, the mRNA ABCA1 expression was augmented to 1.20-fold, 1.88-fold and 2.59-fold, respectively. This study proved that lauric acid and resveratrol were able to elevate ABCA1 gene expression, even though the inhibitors, TNF-α and insulin were present.

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Last but not least, I would like to thank my beloved parents and sisters for their endless love, support, encouragement and understanding which provides me enormous encouragement to complete this project.

DECLARATION

I hereby declare that this project report is based on my original work except for citations and quotations which have been duly acknowledged. I also declare that it has not previously and concurrently submitted or any other degree or award at UTAR or other institutions.

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HIA YI LIN

APPROVAL SHEET

This project entitled "**A STUDY ON THE EFFECTS OF INSULIN, TNF-ALPHA AND ANTIOXIDANTS ON THE EXPRESSION OF ATP-BINDING CASSETTE TRANSPORTER 1 (ABCA1) IN HEPG2 CELLS**" was prepared by HIA YI LIN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (HONS) in Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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Date: _____

PERMISSION SHEET

It is hereby certified that **HIA YI LIN** (ID No: **12ADB06693**) has completed this final year project entitled "**A STUDY ON THE EFFECTS OF INSULIN, TNF-ALPHA AND ANTIOXIDANTS ON THE EXPRESSION OF ATP-BINDING CASSETTE TRASNPORTER 1 (ABCA1) IN HEPG2 CELLS**" supervised by Dr. Chew Choy Hoong (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

Yours truly,

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(HIA YI LIN)

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LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
ABC	ATP-binding cassette
ABCA	ATP-binding cassette transporter subfamily A
ABCA1	ATP-binding cassette transporter subfamily A member 1
Akt/ PKB	Protein kinase B
AP-1	Activator protein
ApoA1	Apolipoprotein A1
ATP	Adenosine triphosphate
BCP	1-Bromo-3-Chloropropane
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CVD	Cardiovascular disease
-d(RFU)/dT	Rate of change of the relative fluorescence units with time
DNA	Deoxyribonucleic acid
DR-4	Direct repeat response element
EDTA	Ethylenediaminetetraacetic acid

et al.	“et alia” (Italia word referring to ‘and other’)
FBS	Foetal bovine serum
FoxO1	Forkhead box protein O1
g	Acceleration of gravity (~9.8 m/s^2)
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
HDL	High density lipoprotein
HepG2	Human hepatocellular carcinoma cell line
IFN-γ	Interferon gamma
IKK	IkappaB kinase
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRS-1	Insulin receptor substrate-1
kb	kilobases
kDA	Kilodalton
LDL	Low density lipoprotein
LXR	Liver X receptor
LXRE	Liver X response elements
LXRα	Liver X receptor alpha
LXRβ	Liver X receptor beta
MAPKs	Mitogen-activated protein kinases

MCFA	Medium chain fatty acid
M-CSF	Macrophage colony stimulating factor
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of rapamycin complex 1
NBD-1	Nucleotide binding domain-1
NBD-2	Nucleotide binding domain-2
NBF	Nucleotide binding folds
NF-κB	Nuclear factor kappa B
nM	Nanomolar
OD	Optical density
PBS	Phosphate buffered saline
PGs	Prostaglandins
PI3K	Phosphatidylinositide 3-kinase
PPAR	Peroxisome proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor γ
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RCT	Reverse cholesterol transport
RFU	Relative fluorescence units
RNA	Ribonucleic acid
ROS	Reactive oxidative species
rRNA	Ribosomal ribonucleic acid
RXR	Retinoic X receptor

Sirt1	Sirtuin 1
SREBP	Sterol regulatory element-binding protein
SREBP-1c	Sterol regulatory element-binding protein-1c
SREBP-2	Sterol regulatory element-binding protein-2
SYBR	Synergy Brands, Inc. (stock symbol)
TAE	Tris-Acetate-EDTA
TGF-β	Transforming growth factor-beta
T_m	Melting temperature
TNF-α	Tumour necrosis factor-α
tRNA	Transfer ribonucleic acid
Tyr	Tyrosine
v/v	Volume/volume
w/v	Weight/volume
β-actin	Beta-actin

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CHAPTER 1

INTRODUCTION

ATP-binding cassette transporter A1 (ABCA1) is one of the members in ABCA subfamily (Broccardo et al., 1999). It acquires energy through ATP hydrolysis process and plays an important role in regulating cholesterol efflux and high density lipoprotein (HDL) synthesis (Wang and Oram, 2001; Walker, et al., 1982). ABCA1 mediates the translocation of phospholipids and cholesterol to extracellular for the formation of HDL. Its expression is tightly regulated at both transcriptional and post-transcriptional levels (Oram, 2003). Transcription process of ABCA1 gene is usually controlled by two major regulators which are liver X receptor (LXR) and retinoic X receptor (RXR). ABCA1 protein is highly produced in macrophages, placental cells, liver cells and lung cells (Langmann et al., 1999). According Timmins, et al. (2005), hepatic ABCA1 is the major contributor of HDL cholesterol in plasma. Mutation in ABCA1 accelerates the progress of atherosclerosis in Tangier disease due to its defects in the cellular efflux of phospholipids and cholesterol to lipid deficit apolipoprotein A1 (apoA1). In addition, it also fails to produce mature HDL from nascent HDL and reduced level of HDL in blood is the most common characteristics for Tangier disease (Oram and Vaughan, 2000). The discovery of Tangier disease due to ABCA1 mutation depicts the importance of ABCA1 in lipid metabolism (Bodzioch et al., 1999). Apart from lipid transporter, ABCA1 also acts as regulator in apoptosis and inflammation process (Nagao, et al., 2011).

In addition, insulin resistance and hyperinsulinemia are the major concerns in atherosclerosis. Low level of HDL in blood is common phenomenon seen in hyperinsulinemia. With the presence of insulin, not only in skin fibroblasts, the cholesterol removal mechanism in macrophages was also counteracted (Brazg and Bierman, 1993; Wybranska et al., 1996). Insulin was found to have impaired effect on ABCA1 protein and also gene expression level (Nonomura, et al., 2011).

In chronic inflammatory condition such as atherosclerosis, a variety of inflammatory mediators, particularly tumour necrosis factor-α, a pro-inflammatory cytokine is produced by macrophages and other cells (Carswell, et al., 1975; Popa, et al., 2007). TNF-α is involved in many metabolic responses. However, precise role of TNF-α in atherosclerotic plaques of human and rodent is not fully elucidated because different outcomes were obtained in different animal models (Chen, et al., 2009). In obesity and type 2 diabetes, amount of TNF-α is elevated and this condition leads to the occurrence of insulin resistance (Rocha, et al., 2014). Several studies showed that administration of TNF-α induced the insulin resistance in muscle, adipose tissue and hepatocytes (Porter, et al., 2002; Xu, et al., 2008). Besides, TNF-α was found to have regulatory function on ABCA1 gene expression and affect the cholesterol efflux mechanism (Wang et al., 2010). In different cell types, TNF-α exerts different actions on ABCA1 mRNA expression. TNF-α inhibits the activity of ABCA1 gene expression in human THP-1 macrophage-derived foam cells and human intestinal cell line Caco-2 and this leads to defect in lipid transport and cholesterol accumulation in the cells (Field, et al., 2010; Mei, et al., 2007). On

the other hand, TNF-α induces and upregulates ABCA1 gene expression in mouse peritoneal macrophage and phagocyte ingested apoptotic cells (Castrillo, et al., 2003; Gerbod-Giannone, et al., 2006).

Lauric acid and resveratrol are the two antioxidants used in this study. They are widely known for their benefits to human health. Although lauric acid is a saturated fatty acid, but it was proven to have anti-atherogenic, antiviral and antibacterial properties in which it helps to maintain good health in human (Dayrit, 2003; Roos, et al., 2001). Besides, moderate consumption of resveratrol could improve the lipid profile in blood and reduce chances of developing cardiovascular disease due to its antioxidant activity and cardioprotective ability in human body (Henry, et al., 2005; Robert, et al., 2006).

Therefore, the objectives of this study were:

a) To identify the impact of different concentrations of insulin on *ABCA1* mRNA expression in HepG2 cell lines.
b) To determine the optimal concentration of insulin for significant changes (downregulation or upregulation) of *ABCA1* expression in HepG2 cell lines.
c) To investigate the effect of insulin and TNF-α in combination in *ABCA1* expression in HepG2 cell lines.
d) To study the expression of *ABCA1* in HepG2 cells treated with antioxidants (lauric acid or resveratrol) and TNF-α-insulin.

CHAPTER 2

LITERATURE REVIEW

2.1 ATP-binding cassette transporter superfamily

ATP-binding cassette (ABC) transporter superfamily is the largest protein family which consists of total 48 members in human. Therefore, there are 48 distinct ABC transporter genes found in the human genome. The proteins are grouped into 7 subclasses based on the similarities of amino acid sequence and arrangements of domains (Hyde et al., 1990). Generally, ABC transporters are made of a pair of ATP-binding domain and two sets of transmembrane domains (Altenberg, 2004; Hyde, et al., 1990). The ATP-binding domains, also known as nucleotide binding folds (NBF), consist of three conserved domains, Walker A, Walker B and C domains. Walker A and B domains are separated by approximately 90 to 110 amino acids and they are commonly found in all ATP-binding proteins. C domains which are located on upstream of the Walker B domains are used to distinguish ABC transporter from other ATP-binding protein because of its specific structure in ABC protein. In ABC proteins, transmembrane domains contain six membrane-spanning α-helices (Hyde, et al., 1990).

ABC transporters help in the transportation of substrates across extracellular or intracellular membranes after acquiring energy from ATP (Altenberg, 2004). They are also responsible in mediating many processes in the cell (Altenberg, 2004). Therefore, mutations of ABC genes leads to some human genetic

disorders such as anaemia, cholesterol and bile acid transport defects, cystic fibrosis, neurological disease and retinal degeneration (Gabriel, et al., 1993; Mosser, et al., 1993). In addition, studies showed that naturally occurring genetic polymorphisms of ABC transporter contribute to different pharmacokinetic and pharmacodynamics of chemotherapy drugs in different individuals (Altenberg, 2004; Dean, et al., 2001). The protein transporters in ABC superfamily are conserved across species because they are essential in defense mechanism against endobiotics as well as xenobiotics (Altenberg, 2004; Dean, et al., 2001). Defects of ABC transporters cause drug toxicity in cells (Altenberg, 2004; Dean, et al., 2001).

2.2 ATP-binding cassette transporter subfamily A member (ABCA)

ABCA subfamily consists of 12 members of full transporters that are subdivided into two groups (Dean, et al., 2001). The members of first group are ABCA1, ABCA2, ABCA3, ABCA4, ABCA7, ABCA12 and ABCA13 (Dean, et al., 2001). Their location of genes were found in six different chromosomes. While for ABCA5, ABCA6, ABCA8, ABCA9 and ABCA10, they are arranged into cluster on chromosome 17q24. They all have 37-38 exons, a special characteristics used to differentiate ABCA1 with 50 exons (Dean, et al., 2001). These genes were speculated to take part in cellular lipid homeostasis in human body. Recent findings proved that ABCA1, ABCA2 and ABCA7 share the same function and require sterol to upregulate their expression (Kaminski, et al., 2000; Kaminski, et al., 2001). Besides, ABCA4 has its own unique function in which it is highly expressed in rod photoreceptors of eyes. It mediates the transportation of vitamin A derivatives to extracellular environment. Hence, mutations of

ABCA genes always leads to lipid metabolism deficiency and Stargardt disease, a retinal degeneration syndrome commonly found in children (Allikments, et al., 1997).

2.3 ATP-binding cassette transporter subfamily A member 1 (ABCA1)

Human ABCA1 gene is found on chromosome 9q31. Within this chromosome, it has approximately 70 kilobases (kb) and consists of at least 49 protein encoding gene (Oram, 2003). ABCA1 is an integral membrane protein, made up of 2,261 amino acids (Dean, et al., 2001). Topological analysis revealed that ABCA1 protein is made up of two similar structures and they are linked covalently (Bungert, et al., 2001; Fitzgerald, et al., 2001). Nucleotide-binding domain in each of these similar structures consist of two conserved peptide motifs, Walker A and Walker B (Bungert, et al., 2001; Fitzgerald, et al., 2001). These two motifs are found in most protein which utilize ATP (Bungert, et al., 2001; Fitzgerald, et al., 2001). Besides, ABCA1 has two sets of transmembrane domains, comprising of 6 membranes spanning α-helices (Bungert, et al., 2001; Fitzgerald, et al., 2001). N-terminus extends from the transmembrane domain and orients into the cytosol (Bungert, et al., 2001; Fitzgerald, et al., 2001). Two large and highly glycosylated extracellular loops linked by cysteine bonds can also be observed in Figure 2.1, showing the topological model of ABCA1.

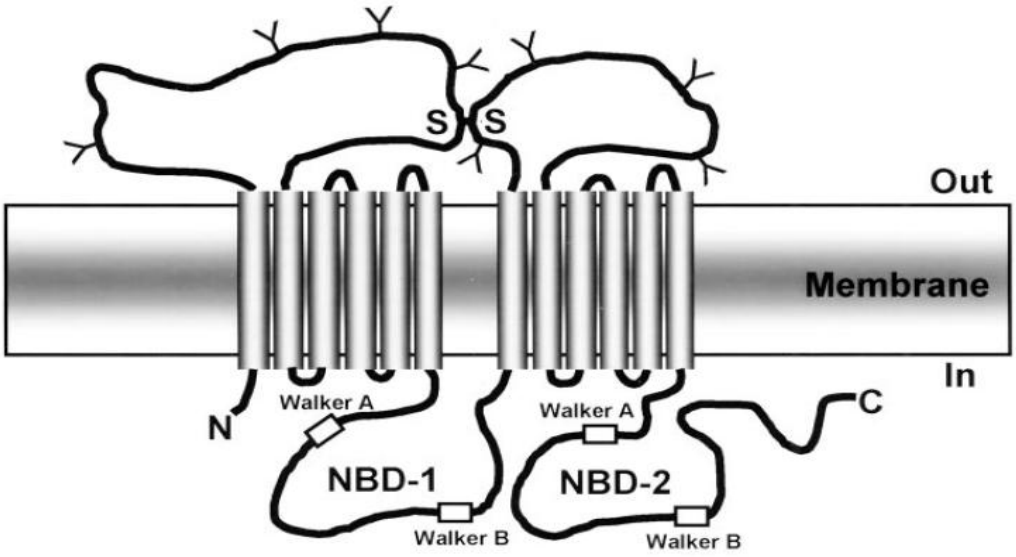


Figure 2.1: Schematic diagram presenting topological model of ABCA1

NBD-1 and NBD-2 represent nucleotide-binding domain which consist of Walker A and Walker B in both domain. C region is the structure used to differentiate ABC transporter from other ATP-binding protein. Two sets of transmembrane domains, 6 membranes spanning α-helices can also be seen from this figure. N terminus penetrates from the membrane into the inner part of cells. Two large and glycosylated loops in the outside membrane of cells are linked by cysteine bonds (Adapted from Oram, 2003).

Products of ABCA1 gene expression can be found in placental, fetal tissues, hepatocytes, lung and adrenal gland (Langmann, et al., 1999). Expression of ABCA1 gene is controlled epigenetically, transcriptionally and also post-transcriptionally (Oram, 2003). ABCA1 gene expression is accelerated by loading cholesterol in the peripheral cells such as macrophages (Lawn, et al., 1999). Ligands such as oxysterols and 9-cis-retinoic acid activate liver X receptor (LXR) and retinoic X receptor (RXR) (Repa and Mangelsdorf, 1999). LXR consists of LXRα and LXRβ. LXRα is only expressed in certain sites such as liver, adrenal glands, kidneys, lungs and adipose tissues whereas LXRβ is expressed in almost all part of body tissues (Bischoff, et al., 2010). In basal level, LXR and RXR binds to liver X response elements (LXREs), also known as direct repeat response element (DR-4) in the promoter region of ABCA1 genes (Bischoff, et al., 2010). When cholesterol builds up in the cells, the binding of oxysterols activates LXR and RXR to start the transcription of ABCA1 (Venkateswaran, et al., 2000). The schematic diagram presenting the regulation of ABCA1 gene expression is shown in Figure 2.2. Furthermore, regulation of ABCA1 activity at its post-translational level is more important to prevent excessive elimination of cholesterol, leading to cell death due to loss of essential components of cell (Wang and Oram, 2001). Therefore, post-translation process is to degrade ABCA1 rapidly and to prevent excessive ABCA1-mediated cholesterol efflux (Wang and Oram, 2001).

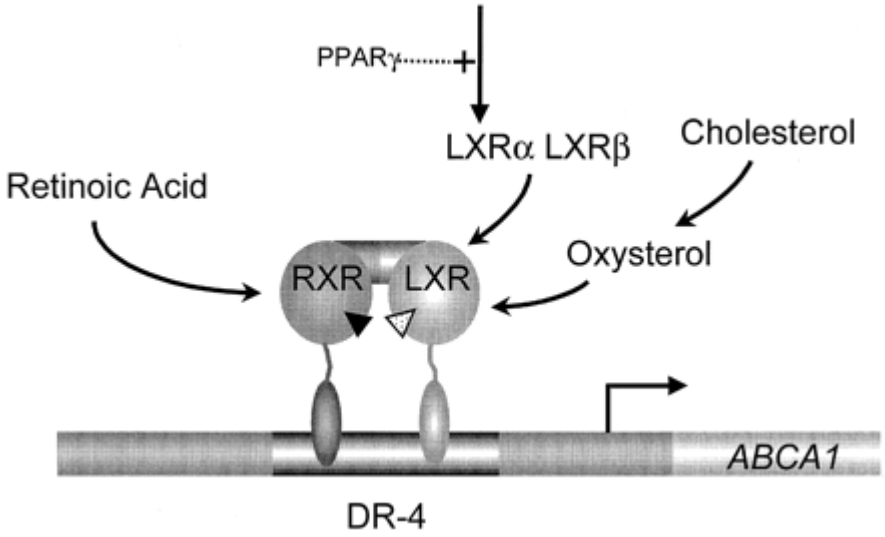


Figure 2.2: Oxysterol, retinoic acid, LXR and peroxisome proliferator-activated receptor (PPAR) activators are the main regulators for ABCA1 gene expression

Ligands such as oxysterol and retinoic acid bind and activate the formation of RXR and LXR heterodimer. Then, this heterodimer induces the ABCA1 gene expression pathway. (Adapted from Oram, 2003)

ABCA1 plays a critical role in lipid homeostasis. ABCA1 is involved in reverse cholesterol transport (RCT), a process where the excess cholesterol is removed from peripheral cells and returned to the liver for excretion (Ohashi, et al., 2005). RCT is important in reversing the progression of atherosclerotic lesion (Ohashi, et al., 2005). ABCA1 is one of the major mediator in RCT in which it expels the free cholesterol and phospholipids to lipid poor apoliprotein, apoAI for the formation of nascent HDL (Fisher, et al., 2012). According to the studies of Tangier disease patients and several animal models, mutations in ABCA1 usually have severe impact on lipid metabolism (Fisher, et al., 2012). Accumulation of sterols can be found in macrophages, hepatocyte and other cells (Fisher, et al., 2012). Therefore, several studies have suggested that ABCA1 is the main factor in the inverse relationship between HDL levels and CVD where-by mutation of ABCA1 leads to poor HDL profile and enhanced progression of atherosclerosis (Liu and Tang, 2011).

2.4 ABCA1 and insulin

Excessive high concentration of insulin in blood leads to atherosclerotic cardiovascular disease. Patients with hyperinsulinemia usually have decreased high-density lipoprotein (Bonora, et al., 2008; Karhapaa, et al., 1994; Zavaroni, et al., 1994) According to Cong, et al. (2014), insulin was found to enhance the degradation rate of ABCA1 protein in completely differentiated 3T3-L1 adipocytes through mediating calpain and proteasome pathway. Besides, insulin has been shown to act as a negative regulator of ABCA1 gene expression in 3T3-L1 adipocytes (Cong, et al., 2014).

Apart from research on 3T3-L1 adipocytres, studies have also been done on human hepatoma cells, HepG2 cells. In HepG2 cells, insulin was proven to have inhibitory effect on ABCA1 protein in which it accelerated the degradation of ABCA1 in protein level without affecting the ABCA1 mRNA levels (Nonomura, et al., 2011). Inhibition of phosphatidylinositide 3-kinase (PI3K) by insulin could be cancelled by inhibitor, for example LY294002 (Nonomura, et al., 2011). LY294002 not only inhibited PI3K, it also acted on other lipid kinases and proteins (Zvelebil, et al., 2007). However, only the degradation of ABCA1 was stopped by the addition of inhibitor but the HDL biogenesis was still suppressed due to phosphorylation of ABCA1 at Tyr1206 leads to reduced ABCA1 activity (Saltiel and Kahn, 2001; Draznin, 2006). In contrast, Sporstsol, et al. (2007) proved that insulin with concentration 10 nm was able to inhibit the mRNA expression of ABCA1 in primary rat hepatocytes according to the results obtained from the qPCR and Western blots.

2.5 Tumour Necrosis Factor –α (TNF-α)

TNF-α is a 17kDa polypeptide which functions as a pleiotropic cell regulatory cytokine (Popa, et al., 2007). TNF-α is produced by several types of body cells, mainly the activated macrophages (Thomas, 2001). Apart from macrophages, TNF-α is also secreted by smooth muscle cells, adipocytes, fibroblast and immune cells such as monocytes and T-cells (Popa, et al., 2007). Its biological activity in the body depends on the cell type it binds to and the presence of the other protein regulators (Carswell, et al., 1975). TNF-α has been widely used as a 'tool drug' to study its roles in cell proliferation, cell death, cell differentiation and most importantly, gene expression (Arner and Langin, 2007). Besides, it also

exerts its biological effects on immune cells such as neutrophils, eosinophils and lymphocytes in inflammatory system (Popa, et al, 2007). Its biological effects can also be seen in lipid metabolism (Arner and Langin, 2007).

Most of the *in vitro* and *in vivo* studies have proven that TNF-α inflammation leads to poor lipid profile in blood (Chen, et al., 2009). As a pro-atherogenic inflammatory cytokines, TNF-α is always positively associated with high triglyceride, total cholesterol and very low density lipoprotein (Jovinge, et al., 1998). High density lipoprotein (HDL) could be hardly found in blood extremely high TNF-α (Jovinge, et al., 1998). In addition, TNF-α is usually found in human and rodent atherosclerotic plaques (Chen, et al., 2009).

However, its roles and exact mechanism in atherosclerosis or some lipid metabolism disorder is not fully elucidated because results from research conducted on different animal models are disputable (Chen, et al., 2009). Molecular defect of ABCA1 in Tangier disease is always linked to severe HDL deficiency and a higher chance of getting atherosclerosis due to accumulation of macrophage foam cells in various tissues. In several studies, ABCA1 was believed to have association with TNF-α (Rigotti and Cohen, 2000). Research has been conducted to verify the effect of TNF-α on ABCA1 gene expression in different human cell lines. In human THP-1 macrophage-derived foam cells, TNF-α inhibited ABCA1 gene and also protein expression level (Mei, et al., 2007). Cellular cholesterol accumulation could be observed after the downregulation of ABCA1 (Mei, et al., 2007). This fact provides an insight that TNF-α might impair cholesterol efflux to form nascent HDL by suppressing

ABCA1 (Mei, et al., 2007). As a result, low HDL in plasma can be observed from patients with activated inflammatory response (Mei, et al., 2007). Besides, ABCA1 was also inhibited by TNF-α in human intestinal cell line Caco-2 (Field, et al., 2010). TNF-α repressed ABCA1 gene expression by decreasing ABCA1 promoter activity and thus reduced the cholesterol efflux to lipid free apoAI (Field, et al., 2010). It also enhanced the ABCA1 degradation rate which further decreased the number of ABCA1 transporter in Caco-2 (Field, et al., 2010). The intestine is also one of the major sources of producing plasma HDL and hence defects of ABCA1 in intestine leads to accumulation of cholesterol (Brunham, 2006). Nevertheless, TNF-α exhibited different action in mouse peritoneal macrophage and in apoptotic cells ingested by phagocytes in which it upregulated ABCA1 (Castrillo, et al., 2003; Gerbod-Giannone, et al., 2006). Therefore, researchers have proven that the pro-atherogenic or anti-atherogenic roles of TNF-α heavily depends on the type of cells involved, the specific receptors and the downstream signalling pathways mediated by TNF-α (Chen, et al., 2009).

2.6 Lauric acid

Lauric acid is natural saturated fatty acid and beneficial medium chain fatty acid (MCFA) with 12 carbon atom chain (Glade, 2000). Coconut oil and palm kernel oil contains more than 40% of lauric acid compared to other types of oil (Denke and Grundy, 1992; Glade, 2000). Lauric acid and monolaurin, a monoester metabolised from lauric acid are very effective in antibacterial and antiviral activity (Lieberman, Enig and Preuss, 2006). Lauric acid has greater ability in killing bacteria compared to other medium chain fatty acid such as capric acid

with 10 carbon atom chain (Lieberman, Enig and Preuss, 2006). Lauric acid kills the bacteria or virus through dissolving the lipids and phospholipids present in the outer layer of pathogen (Projan, et al., 1994). As a consequence, the envelope of pathogen is no longer intact and lauric acid causes the death of pathogen (Projan, et al., 1994). Besides, lauric acid also interferes with the signal transduction in cell replication and prevents the growth of bacteria (Projan, et al., 1994). Moreover, it is claimed that able to reduce the viral load in the HIV patients through inactivation of HIV virus (Lieberman, Enig and Preuss, 2006).

Although lauric acid is a saturated fatty acid, studies showed that it did not elevate LDL-cholesterol concentration due to its poor hypercholesterolemic effects (Roos, et al., 2001; Siri-Tarino, et al., 2010). In fact, HDL cholesterol in serum increased significantly with the consumption of lauric acid and a more favourable serum lipoprotein pattern was observed (Roos, et al., 2001; Siri-Tarino, et al., 2010). As a medium chain fatty acid, it is usually catabolised rapidly in the liver and converted into energy (Dayrit, 2003). It is seldom deposited in adipose tissues and hence lauric acid is not a contributing factor for atherosclerosis although it is a saturated fatty acid (Dayrit, 2003). According to Cheah, et al. (2014), lauric acid could reverse the suppression effect of TNF-α on expression of ABCA1 in HepG2 cells through increasing the LXRα activity which is a positive regulator for ABCA1 gene expression. Besides, lauric acid also shows anti-inflammatory properties which can alleviate atherosclerosis by inhibiting the activation of pro-inflammatory cytokine such as IFN-γ and reduce the inhibition effect by IFN-γ on induction of ABCA1 gene expression (Lim, et al., 2015).

2.7 Resveratrol

Resveratrol or 3, 5, 4'-trihydroxystilbene, is a plant derived polyphenol mainly found in red wines (Soleas, Diamandis and Goldberg, 1997). It is readily absorbed in the human digestive system and commonly consumed as dietary supplements or traditional medicines (Henry, et al., 2005). Dietary consumption of resveratrol can help to maintain human health (Henry, et al., 2005). Resveratrol is claimed to possess antioxidant ability, prevent LDL oxidation in human body and also apoptosis induced by oxidants (Robert, Joshua and David, 2006). Besides, high consumption of wine was correlated with decreased prevalence of coronary heart disease due to the cardioprotective ability of resveratrol found in red wines (Robert, Joshua and David, 2006).

Antioxidant power of resveratrol plays important role in delaying oxidative stress induced by cellular damage (Robert, Joshua and David, 2006). Besides, it has been shown to exhibit ability in preventing oxidative-induced apoptosis in several cell lines, such as Swiss 3TS mouse fibroblasts and human peripheral blood mononuclear (Kutuk, et al., 2004; Losa, 2003). Reactive oxidative species (ROS) produced by activated platelets during early atherosclerosis was also inhibited by resveratrol (Olas and Wachowicz, 2002). Therefore, the antioxidant activity of resveratrol is always associated with the prevention of development of atherosclerosis.

According to Constant (1997), although the French population has high fat food consumption, the incidence of coronary heart disease is unexpectedly low in their population. Studies showed that this may be associated with high daily

consumption of red wine (Constant, 1997). Resveratrol protects the cardiovascular system by inhibiting platelet aggregation because excessive aggregation of activated platelet in vascular system can promote the development of cardiovascular related disease (Bhat, et al., 2001). Besides, resveratrol also exerts vasorelaxation effect on rat aortic rings, helping to relieve high blood pressure in blood vessels (Chen and Pace-Asciak, 1996). Prevention of LDL oxidation by resveratrol also improves the blood lipid profile in human body (Frankel, Waterhouse and Kinsella, 1993; Fremont, Belguendouz and Delpal, 1999).

Anticancer activity of resveratrol was explored and it was discovered that it can inhibit tumour formation in initiation, promotion and progression stage. It is involved in arresting the cell cycle to inhibit the proliferation of mutated cells (Castello and Tessitore, 2005). Besides, proliferation of cancer cell lines can also be inhibited through apoptosis mechanism performed by resveratrol (Wolter, et al., 2002). Activation of p53 and the inhibition of NF-κB and activator protein-1 (AP-1) were the proposed molecular mechanisms utilised by resveratrol in order to inhibit the uncontrolled growth of cancer cell lines (Banerjee, Bueso-Ramos and Aggarwal, 2002; Huang, et al., 1999; Kundu, et al., 2004).

Moreover, resveratrol also prevents inflammation by reducing prostaglandins (PGs) activity in inflammatory process (Richard, et al., 2005). At first, resveratrol suppresses the expression of cyclooxygenase-2 (COX-2), an enzyme which helps in PG formation. Reduced level of COX-2 decreases the level of PG and thus the inflammation effect subsides (Martin, et al., 2004; Richard, et al.,

2005). In addition, cytokines which are involved in mediating inflammatory action such as TNF-α and interleukin-1β (IL-1β) are also suppressed by resveratrol (Culpitt, et al., 2003). Inhibition of TNF-α induced NF-κB also can be achieved by resveratrol to reduce inflammation response (Holmes-McNary and Baldwin, 2000; Manna, Mukhopadhyay and Aggarwal, 2000). Resveratrol prevents the nuclear translocation of the NF-κB subunit p65 by arresting phosphorylation of the subunit (Manna, Mukhopadhyay and Aggarwal, 2000). Blocking of NF-κB DNA binding and suppressing IκB kinase (IKK). IκB, an inhibitory protein was not phosphorylated by deactivated IKK and it was able to bind to inactive NF-κB, further suppresses the NF-κB mechanism and stops the occurrence of inflammatory process (Holmes-McNary and Baldwin, 2000).

CHAPTER 3

MATERIALS AND METHODS

3.1 Stock solutions

Stock solutions of 10 × TAE buffer used for RNA electrophoresis was prepared according to Table 3.1.

Table 3.1: Composition of solution for RNA electrophoresis

Solution	Composition
10 × TAE buffer	0.5 M EDTA, 0.2 M Glacial acetic acid, 0.4 M Tris-base, sterile deionised distilled water was used to top up to 1L.

3.2 Preparation of glassware and plasticware

Before using them, all glassware and plasticware (pipette tips and microcentrifuge tubes) were autoclaved at 121 ℃ for 15 minutes and the pressure at 975 kPa. Deionised distilled water was also autoclaved prior to use.

3.3 Cell culture techniques

3.3.1 Maintenance of cells in culture

Together with 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, USA), minimum essential medium (Gibco, USA) was used to grow HepG2 cells (American Type Culture Collection, USA). HepG2 cells in 75 cm^2 tissue culture flasks were maintained in a humid incubator of 5% (v/v) CO_2 at 37 ℃. The cell culture medium was observed for any colour changes (an indicator for contamination) before the flasks were replaced with new medium every three days.

3.3.2 Subculturing of cells

Passaging of cells was carried out after the cells have achieved 80% confluence. Initial medium was discarded by using sterile serological pipette. After this, the cells were washed twice with 5 ml of phosphate buffered saline (PBS) for 75 cm^2 flasks and 2 ml of PBS (Calbiochem, UK) for 25 cm^2 flasks in order to remove trypsin inhibitors in the medium containing serum.

Then, 2 ml of trypsin for 75 cm^2 flask or 1 ml for 25 cm^2 flasks was added. Trypsinisation was required to disrupt the cell-substratum and cell-cell interactions. The flasks were then incubated for 10 to 15 minutes in humid incubator of 5% (v/v) CO_2 at 37 ℃. After incubation, trypsinisation was stopped by putting 4 ml of minimum essential medium to dilute the trypsin (Gibco, USA). The diluted trypsin was transferred to a 50 ml of centrifuge tube and centrifuged at 800 g for 8 minutes. The purpose of centrifugation was to pellet the cells. Supernatant and pellet were obtained after centrifugation. Next, the supernatant

was removed and 1.5 ml of MEM was added to the pellet. Together with the pellet, medium inside the centrifuge tubes were resuspended gently to ensure the even distribution of the cells.

3.4 Treatment of HepG2 cells

3.4.1 Treatment of HepG2 cells with insulin

HepG2 cells were grown in 25 cm^2 tissue culture flasks. When they reached 60% confluence, they were treated with different types of stimulants. Before treating the cells, the medium was discarded and the flasks were washed twice with PBS. Fresh medium supplemented with 0.5% (v/v) FBS were added to the flask after the washing step. The cells were then treated with different concentrations of insulin (Nacalai Tesque, Japan). The concentration of insulins added were 1 nM, 5 nM, 10 nM, 50 nM and 100 nM, respectively. After this, the cells were incubated in 5% (v/v) CO_2 at 37 ℃ for 24 hours.

3.4.2 Treatment of HepG2 cells with insulin, TNF-α and lauric acid

Optimum concentration of insulin from first experiment (Section 3.4.1) was then used in the subsequent experiment. For this, a combination of insulin, TNF-α (Merck Millipore, Germany) and lauric acid (Sigma-Aldrich, USA) were used. The concentration of TNF-α used was 20 ng/ml in accordance to the concentration used in previous studies where treatment with 20ng/ml of TNF-α was ample to suppress PPARα mRNA expression significantly (Lim et al., 2013). Nonetheless, with concentration exceeded 20 ng/ml of TNF-α, only mild suppression effect on mRNA expression (Lim et al., 2013). While, the concentration of lauric acid added were in a dose dependent manner 5 μM, 10

μM and 20 μM. According to Wong and Chew (2015), increasing concentration of lauric acid improved the cell viability. However, excessive high concentration of lauric acid will affect the fatty acid catabolism activity and therefore the ideal and safe dosage of lauric acid for this experiment were 5 μM, 10 μM and 20 μM (Wong and Chew, 2015). After exposed to the treatment, the cells were kept set at 5 % (v/v) CO_2 incubator at 37 ℃ for 24 hours.

3.4.3 Treatment of HepG2 cells with insulin, TNF-α and resveratrol

In third experiment, resveratrol (ChromaDex, California) was used instead of lauric acid. Five nanomolar of insulin and 20 ng/ml of TNF-α were used in this set of treatment while resveratrol added was in the range of 5 μM, 10 μM and 20 μM. The cells were then incubated at 5 % (v/v) CO_2 at 37 ℃ for 24 hours before RNA extraction was carried out. For all treatment, untreated samples were only exposed to MEM and FBS.

3.5 RNA-Associated Techniques

3.5.1 Isolation of total cellular RNA using Tri-Reagent® LS

Tri-Reagent® LS (Molecular Research Centre, Singapore) was used to isolate the total cellular RNA from the cells culture in 25 cm^2 tissue culture flasks. According to the manufacturer's instruction, 0.75 ml of Tri-Reagent® LS was required for every 0.25 ml of sample. After adding Tri-Reagent® LS to the sample, the cells were scraped using cell scraper (TPP, USA). Pipetting step was performed repetitively to allow lysis of cells. The cell suspension was then transferred into 1.5 ml sterile microcentrifuge tubes and these tubes were left at room temperature for 5 minutes for complete dissociation of nucleoprotein

complexes. A volume of 0.1 ml of 1-Bromo-3-Chloropropane (BCP) (Sigma-Aldrich, USA) was added into the tubes and the tubes were shaken vigorously for 15 seconds. Centrifugation was performed at 12000 *g* for 15 minutes at 4 ℃ after the tubes were incubated in room temperature for 15 minutes. After centrifugation, a colourless upper aqueous phase was obtained. This aqueous phase which consisted of RNA was pipetted to a new microcentrifuge tube. To precipitate RNA, 0.5 ml of isopropanol (Merck KgaA, Germany) was added into the tubes and the mixture was left at room temperature for 10 minutes. After this, the mixture was centrifuged at 16000 *g* for 5 minutes at 4 ℃. The supernatant was discarded before the RNA pellet was washed with 1 ml of 75% of ethanol. RNA pellet together with the ethanol was centrifuged again at 16000 *g* for 5 minutes at 4 ℃. The RNA pellet was air-dried for 5 minutes. Subsequently, the pellet was resuspended in 30 µl of sterile deionised water, the mixture was mixed thoroughly and the tubes were incubated in water bath with temperature at 55 ℃ for 15 minutes.

3.5.2 Electrophoresis of RNA on 1% denaturing agarose-bleach gel

The modified protocol from Aranda et al. (2012) was used for this purpose. Electrophoresis of RNA was carried out using 1 % (w/v) denaturing agarose-bleach gel. Specifically, 0.2 g of agarose powder (Choice-Care Sdn. Bhd., Malaysia) was dissolved in 200 ml of 1× TAE buffer. The agarose was then incubated with 400 µl of bleach (Tesco brand) for 5 minutes. The mixture was heated to dissolve agarose powder completely. This mixture was poured into a gel-casting tray after the agarose solution was cooled to 55 ℃ and then a comb was inserted properly. The gel was solidified, the samples were loaded with

RNA loading dye in the ratio of 3:1 (3 µl of RNA loading dye to 1 µl of sample). The gel tank was filled with 1×TAE as the electrophoresis running buffer.

3.5.3 Spectrophotometric measurement of RNA

NanoDrop spectrophotometer (Thermo Scientific) was used to measure the optical density at wavelength 230 nm, 260 nm and 280 nm. The ratio of optical density at 260 nm and 280 nm was used to check the purity of extracted total cellular RNA. Besides, ratio 260 to 230 was accessed because it also acted as a secondary measure showing the purity of RNA.

3.6 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

3.6.1 Primers sequence for ABCA1 and β-actin used in qRT-PCR

The nucleotide sequence of ABCA1 and β-actin primers used in qRT-PCR are presented in Table 3.2.

Table 3.2: Nucleotide sequence of primers used in qRT-PCR

Primer	Primer sequence	Length
ABCA1 Forward	GCACTGAGGAAGATGCTGAA	20 mers
ABCA1 reverse	AGTTCCTGGAAGGTCTTGTT	20 mers
β-actin Forward	TCACCCTGAAGTACCCCATC	20 mers
β-actin Reverse	CCATCTCTTGCTCGAAGTCC	20 mers

(Adapted from Chew et al., 2007)

3.6.2 Relative quantification of ABCA1 mRNA expression through qRT-PCR

Quantitative RT-PCR was performed by using QuantiTect SYBR Green RT-PCR Master Mix (QIAGEN, Germany) in order to quantify ABCA1 the mRNA expression. All master mix reactions were prepared with final volume of 20 µl on ice. The components are shown in Table 3.3.

The samples were placed in CFX96^{TM} Real-Time PCR Detection System (Bio-Rad, USA) for relative quantitative PCR (qPCR). In order to determine the relative mRNA expression, the quantity of mRNA target was normalised against β-actin, a housekeeping gene which acted as an internal control. Each experiment was run in triplicate for each gene.

Table 3.3: Components of qRT-PCR for ABCA1 (QIAGEN)

Components	Volume (µl)
2×QuantiTect SYBR Green PCR Master Mix	10
Deionised distilled water	6.8
Forward Primer	1.0
Reverse Primer	1.0
Reverse Transcriptase	0.2
RNA template (50 ng/µl)	1.0
Total volume	20

Table 3.4: Protocol for the qRT-PCR Quantification of ABCA1 mRNA Amplification

Step	Dwell Time	Set point	Activity	Cycle	PCR/Melt Data acquisition	Temperature change
1	30 min	30 ℃	Reverse Transcription	1		
2	15 min	95 ℃	PCR initial activation step	1		
3	15 sec	94 ℃	Denaturation	35		
	30 sec	60 ℃	Annealing			
	30 sec	72 ℃	Extension			
4	1 min	95 ℃		1		
5	1 min	55 ℃		1		
6	1 min	65 ℃		81	Melt Curve	0.5 ℃, increment every 10 sec

3.7 Statistical analysis

Student's t-test in SPSS (version 20) software was utilized for statistical analysis.

$p<0.05$ was considered as statistically significant.

CHAPTER 4

RESULTS

4.1 HepG2 cells culture

Originally, HepG2 cells was obtained from a hepatoblastoma of liver tissue in a 15 years old Caucasian male (Knowles et al., 1980). These cells are adherent cells and they are epithelial in morphology as shown Figure 4.1 (Knowles et al., 1980). The chromosome of this hepatoblastoma consists of an incomplete region in 4q3 (Tomlinson et al., 2005). Besides, it also consists of abnormal chromosomal number, trisomies in chromosome 2 and 20 (Tomlinson et al., 2005). In this project, HepG2 cells were grown in complete medium which made up of foetal bovine serum and minimum essential medium as described in Section 3.3.1.

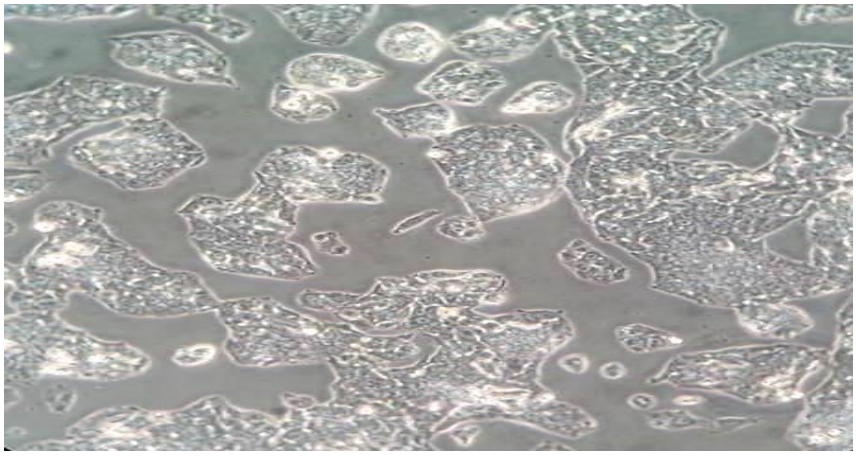


Figure 4.1: Human hepatocellular carcinoma cell, HepG2 cells (Magnification: 400 ×)

4.2 Isolation of total cellular RNA

When the cells reached around 60%, they were treated with different types of stimulants as illustrated in Section 3.4. At first, HepG2 cells were treated with different concentration of insulin. In second set and third set of experiment, the cells were exposed to insulin with TNF-α and lauric acid or resveratrol (5 μM, 10 μM, 20 μM) for 24 hours.

After each set of experiment, extraction of total cellular RNA was performed using Tri-Reagent® LS, following the procedure provided by manufacturer (Section 3.5.1). Then, the extracted total cellular RNA were sub-aliquoted to three sets and one set was subjected to 1% denaturing agarose-bleach gel electrophoresis. One μl of isolated RNA was subjected to 1% denaturing agarose-bleach gel for the purpose of determining the integrity of extracted total cellular RNA (Section 3.5.2). The image of denaturing agarose-bleach gel electrophoresis are as shown in Figure 4.2, Figure 4.3 and Figure 4.4. Two thick and distinct bands, 28 rRNA and 18 ribosomal RNA (rRNA) could be observed and it indicated the intact integrity of extracted total RNA.

Besides, spectrophotometric measurement was also carried out to access the purity of extracted total cellular RNA. The ratio of optical density (OD) at 260 nm and 280 nm within a range of 1.8 to 2.0 represents that the RNA was free from any contamination. (Tables 4.1-4.3)

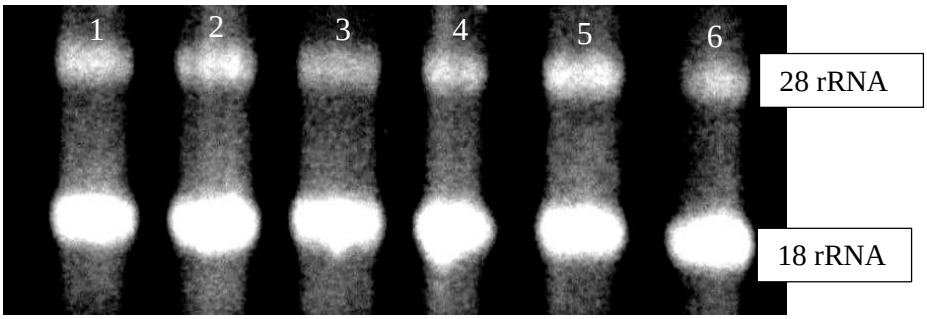


Figure 4.2: Denaturing agarose-bleach gel electrophoresis of total cellular RNA isolated from HepG2 cells after insulin treatment in different concentration

Three µl of RNA loading dye and 1 µl of extracted total cellular RNA were subjected to 1% (w/v) denaturing agarose-bleach gel electrophoresis.

Lane 1: RNA from untreated HepG2 cells.

Lane 2: RNA from HepG2 cells treated with 1 nM of insulin.

Lane 3: RNA from HepG2 cells treated with 5 nM of insulin.

Lane 4: RNA from HepG2 cells treated with 10 nM of insulin.

Lane 5: RNA from HepG2 cells treated with 50 nM of insulin.

Lane 6: RNA from HepG2 cells treated with 100 nM of insulin.

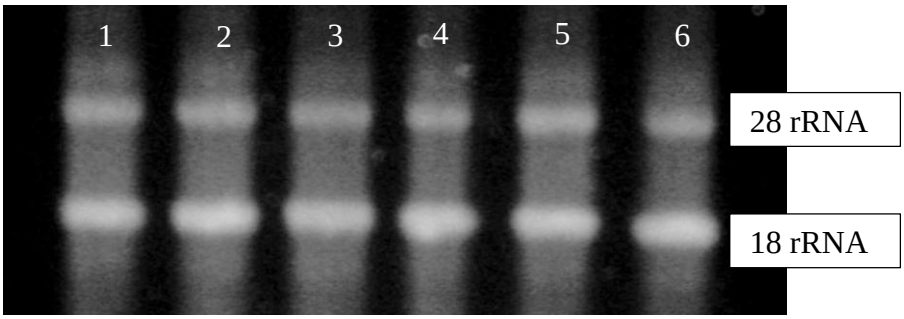


Figure 4.3: Denaturing agarose-bleach gel electrophoresis of total cellular RNA isolated from HepG2 cells after treatment with 5 nM of insulin and 20 ng/ml of TNF-α with lauric acid

Three µl of RNA loading dye and 1 µl of extracted total cellular RNA were subjected to 1% (w/v) denaturing agarose-bleach gel electrophoresis.

Lane 1: RNA from untreated HepG2 cells.

Lane 2: RNA from HepG2 cells treated with 5 nM of insulin.

Lane 3: RNA from HepG2 cells treated with 5 nM of insulin and 20 ng/ml of TNF-α.

Lane 4: RNA from HepG2 cells treated with 5 nM of insulin, 20 ng/ml of TNF-α and 5 µM of lauric acid.

Lane 5: RNA from HepG2 cells treated with 5 nM of insulin, 20 ng/ml of TNF-α and 10 µM of lauric acid.

Lane 6: RNA from HepG2 cells treated with 5 nM of insulin, 20 ng/ml of TNF-α and 20 µM of lauric acid.

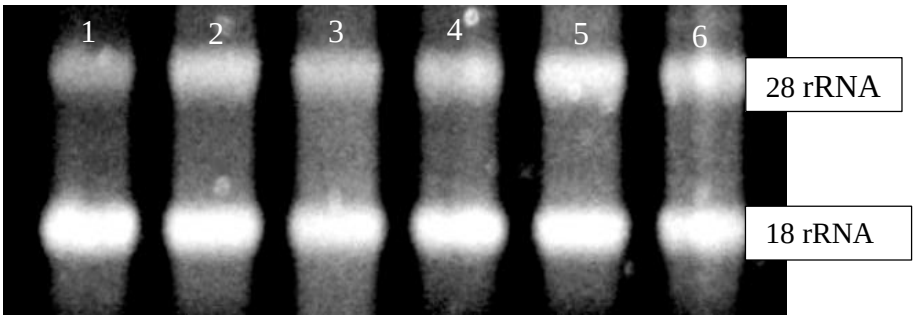


Figure 4.4: Denaturing agarose-bleach gel electrophoresis of total cellular RNA isolated from HepG2 cells after treatment with 5 nM of insulin and 20 ng/ml of TNF-α with resveratrol

Three µl of RNA loading dye and 1 µl of extracted total cellular RNA were subjected to 1% (w/v) denaturing agarose-bleach gel electrophoresis.

Lane 1: RNA from untreated HepG2 cells.

Lane 2: RNA from HepG2 cells treated with 5 nM of insulin.

Lane 3: RNA from HepG2 cells treated with 5 nM of insulin and 20 ng/ml of TNF-α.

Lane 4: RNA from HepG2 cells treated with 5 nM of insulin, 20 ng/ml of TNF-α and 5 µM of resveratrol.

Lane 5: RNA from HepG2 cells treated with 5 nM of insulin, 20 ng/ml of TNF-α and 10 µM of resveratrol.

Lane 6: RNA from HepG2 cells treated with 5 nM of insulin, 20 ng/ml of TNF-α and 20 µM of resveratrol.

Table 4.1: Concentration and A_{260}/A_{280} ratio of total RNA from HepG2 cells treated with different concentrations of insulin

Treatment	A_{260}	A_{280}	Ratio of A_{260}/A_{280}	Concentration of RNA (ng/µl)
Without treatment	54.396	27.333	1.99	2175.83
1 nM insulin	57.631	29.100	1.98	2305.25
5 nM insulin	45.267	22.656	2.00	1810.67
10 nM insulin	45.698	22.890	2.00	1827.92
50 nM insulin	53.073	26.597	2.00	2122.92
100 nM insulin	46.216	22.998	2.01	1848.63

Table 4.2: Concentration and A_{260}/A_{280} ratio of total RNA from HepG2 cells treated with 5 nM of insulin, 20 ng/ml of TNF-α and different concentrations of lauric acid

Treatment	A_{260}	A_{280}	Ratio of A_{260}/A_{280}	Concentration of RNA (ng/µl)
Without treatment	35.375	17.496	2.02	2175.83
5 nM insulin only	54.819	27.272	2.01	2305.25
5 nM insulin, 20 ng/ml TNF-α	34.810	17.164	2.03	1810.67
5 nM insulin, 20 ng/ml TNF-α, 5 µM lauric acid	27.472	13.435	2.04	1827.92
5 nM insulin, 20 ng/ml TNF-α, 10 µM lauric acid	28.069	13.759	2.04	2122.92
5 nM insulin, 20 ng/ml TNF-α, 20 µM lauric acid	26.610	13.162	2.02	1848.63

Table 4.3: Concentration and A_{260}/A_{280} ratio of total RNA from HepG2 cells treated with 5 nM of insulin, 20 ng/ml of TNF-α and different concentrations of resveratrol

Treatment	A_{260}	A_{280}	Ratio of A_{260}/A_{280}	Concentration of RNA (ng/µl)
Without treatment	40.961	20.278	2.02	1638.44
5 nM insulin only	47.538	23.802	2.00	1901.53
5 nM insulin, 20 ng/ml TNF-α	45.330	22.398	2.02	1813.20
5 nM insulin, 20 ng/ml TNF-α , 5 µM resveratrol	31.699	15.540	2.04	1267.96
5 nM insulin, 20 ng/ml TNF-α, 10 µM resveratrol	36.847	18.162	2.03	1473.86
5 nM insulin, 20 ng/ml TNF-α, 20 µM resveratrol	35.653	17.424	2.05	1426.14

4.3 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

4.3.1 PCR amplification for ABCA1

Quantitative RT-PCR is time-saving, automated, sensitive and high throughput method to quantify of the mRNA expression compared to standard PCR method. During the PCR process, qRT-PCR is able to identify amplified sequence simultaneously. Amplification of the product is measured by qRT-PCR during the exponential phase. Besides, entire process of qRT-PCR including analysis can be carried out inside the same tube.

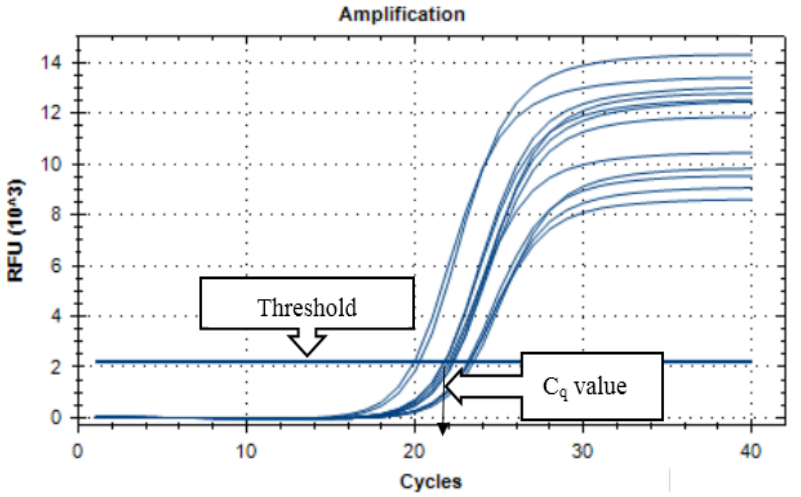
Therefore, this techniques was employed in this study by using QuantiTect SYBR Green RT-PCR Master Mix as described Section 3.6 to examine and quantify the mRNA expression profile of the effect of lauric acid and resveratrol on ABCA1 in human hepatoblastoma, HepG2 cells. Gene expression analysis was automated calculated by software in CFX96^{TM} Real-Time PCR Detection System. All extracted RNA samples were repeated three times for both ABCA1 genes and housekeeping gene to provide significant and accurate data as to ensure the reliability. β-actin was used as the housekeeping gene in this studies which acted as a reference for each normalisation.

The amplification curves of ABCA1 and β-actin are presented in Figure 4.5. The X-axis on amplification plot represents the number of PCR cycles while the Y-axis renders the relative fluorescence units (RFU) which is the fluorescence intensity of the reporter dye. A threshold level, an arbitrary level of fluorescence was chosen above the baseline. The number of PCR required for the reporter

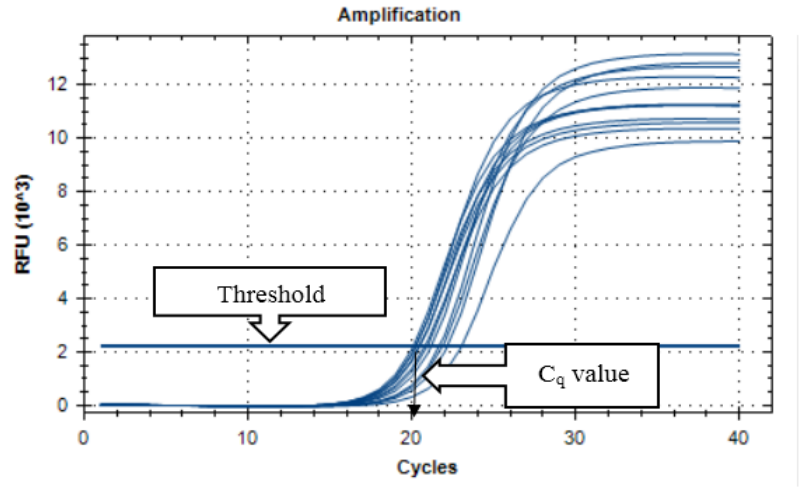
fluorescence to reach the threshold, C_q value was also determined and labelled on the Figure 4.5.

4.3.2 Melting curve analysis

Melting curve chart analysis is important to ensure the target specificity of primer before proceed to analysis of dose response effect of ABCA1 expression which the HepG2 cells were stimulated by insulin, TNF-α and antioxidants (lauric acid and resveratrol). Melting curve was obtained when the amplification cycles were completed. The change in fluorescence was measured immediately once the temperature rose by one fraction of a degree. The steep decreasing slope was due to double-stranded DNA separated at the melting point. The software in thermal cycler program plotted the rate of change of relative fluorescence unit (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature (Celsius) along the X-axis. The peak in the melting curve represents the melting temperature (T_m) of the amplified product. As shown in Figures 4.6, the melting temperature for ABCA1 was 83.50°C while the melting temperature for β-actin was determined as 88.50 ℃. The single peak shown in the melting curve proved that the PCR amplification was successful and the qRT-PCR was specific.



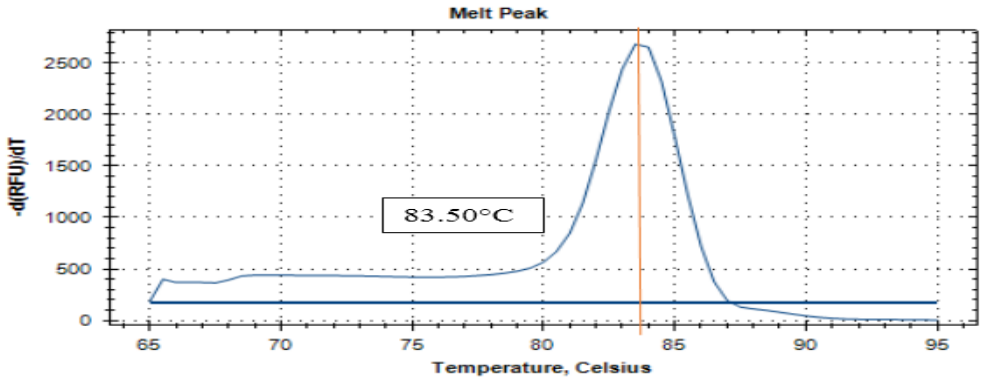
(a)



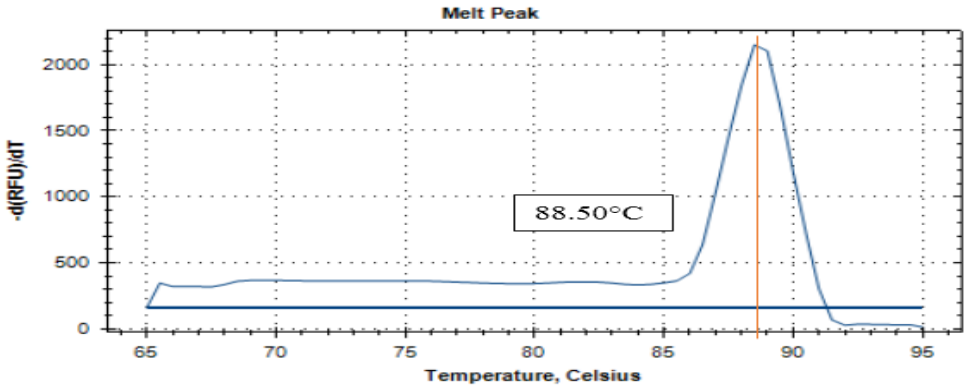
(b)

Figure 4.5: Amplification plot from qRT-PCR of (a) ABCA1 genes and (b) β-actin genes

This PCR amplification plot shows the accumulation of fluorescence intensity in a sigmoid curve. The cycle number is situated in the X-axis, while the arbitrary fluorescence unit (RFU) is shown in the Y-axis. Threshold value is set above the baseline value and the cycle number required to reach threshold is also clearly pointed out.



(a)



(b)

Figure 4.6: Graphical representation of a melting peak analysis in qRT-PCR for (a) ABCA1 genes and (b) β-actin genes

The automated qRT-PCR system carried out melt peak analysis after PCR amplification. The change of fluorescence intensity was measured and recorded as the temperature changed by one fraction of degree Celsius. A steep slope after the melting point is shown in which indicated that the double-stranded DNA separated at the melting point and the fluorescence intensity diminished rapidly. The rate of change of relative fluorescence unit (RFU) with time versus temperature in degree Celsius graph was plotted automatically by the software. The peaks represent the melting temperature (T_m) for the PCR product from (a) ABCA1, which point was at 83.50 ℃ and (b) β-actin, which was at 88.5 ℃.

4.4 The dose response effect

4.4.1 The dose response effect of insulin on ABCA1 mRNA level

At first, HepG2 cells were stimulated with different concentrations of insulin (Section 3.4.1). Untreated HepG2 cells were used as the negative control with a normalised gene expression value of 1.00. The fold value of ABCA1 mRNA was normalised to β-actin and relative to the expression of HepG2 cells. Overall, ABCA1 mRNA expression showed a decreasing pattern in a dose dependent manner. In 1 nM of insulin, the expression was suppressed to 0.46-fold, significantly. With the addition of higher concentration, 5 nM, 10 nM and 50 nM of insulin, ABCA1 gene expression was decreased to about 0.30-fold while 100 nM of insulin further diminished the expression to 0.26-fold. All these decrements were considered statistically significant and shown in Figure 4.7.

4.4.2 The dose response effect of lauric acid on ABCA1 mRNA expression in insulin together with TNF-α treated HepG2 Cells

In the second experiment, HepG2 cells were exposed to insulin, TNF-α and various concentrations of lauric acid (Section 3.4.2). As seen in Figure 4.8, 5 nM of insulin was able to suppress the mRNA expression by 0.29-fold, which was a significant suppression. In the presence of TNF-α, the ABCA1 mRNA expression was further downregulated to 0.20-fold. However, the presence of lauric acid in insulin and TNF-α treated cells upregulated the expression. Lauric acid increased ABCA1 expression in a dose-dependent manner to 1.05-fold, 1.22-fold and 1.72-fold, with 5 μM, 10 μM and 20 μM of lauric acid co-treatment, respectively. All the upregulation of ABCA1 by different

concentrations lauric acid were statistically significant when compared to insulin with TNF-α-treated HepG2 cells.

4.4.3 The dose response effect of resveratrol on ABCA1 mRNA expression in insulin together with TNF-α treated HepG2 Cells

In section 3.4.3, HepG2 cells were treated with insulin, TNF-α and resveratrol with a range of concentration. For this experiment, the effect of insulin alone and insulin with TNF-α on ABCA1 were verified once again in Figure 4.9. Co-treatment with resveratrol proved it produced the same effect as lauric acid. ABCA1 expression was elevated gradually to 1.20-fold, 1.88-fold and 2.59-fold when insulin-TNF-α treated HepG2 cells were stimulated with 5 μM, 10 μM and 20 μM of resveratrol respectively. Again, ABCA1 expression was elevated significantly according to statistical analysis.

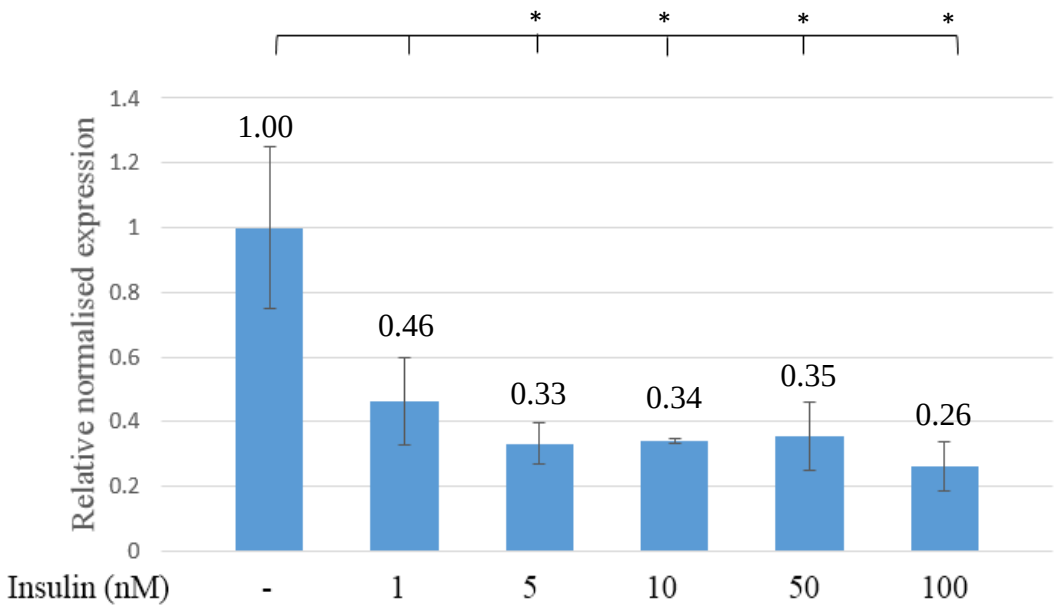


Figure 4.7: Graphical representation showing different dose response of ABCA1 expression in insulin treated HepG2 cells

This gene expression chart of ABCA1 was analysed by the program in CFX96™ Real-Time PCR Detection System. The concentration of insulin (nM) is indicated on X-axis, while the relative normalised gene expression is represented on Y-axis. The value above each bar chart shows the fold value of ABCA1 gene expression which has been normalised to β-actin and compared with the negative control (untreated HepG2 cells). The value of relative normalised expression for untreated sample which was assigned as 1.00. Error bars in each bar chart represents the standard deviation. Student's *t* test (two-sided) was used for statistical analysis. * indicates $p<0.05$ which represents statistically significant change from untreated HepG2 cells.

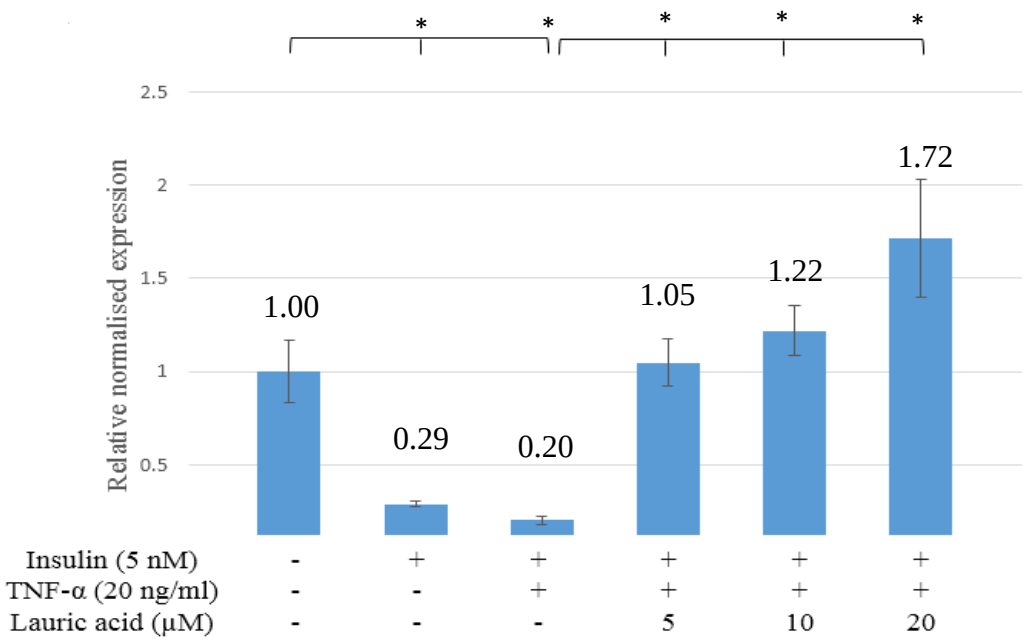


Figure 4.8: Graphical representation showing dose response of ABCA1 expression in HepG2 cells stimulated by 5 nM insulin, 20 ng/ml TNF-α and 5 μM, 10 μM and 20 μM of lauric acid

This gene expression chart of ABCA1 was analysed by the program in CFX96^{TM} Real-Time PCR Detection System. The concentration of lauric acid is indicated on X-axis, while the relative normalised gene expression is represented on Y-axis. The value above each bar chart was interpreted as the fold value of ABCA1 gene expression which has been normalised to β-actin and compared with the negative control (untreated HepG2 cells). Student's *t* test (two-sided) was used for statistical analysis. * indicates $p<0.05$ which represents statistically significant change from untreated HepG2 cells or TNF-α-treated HepG2 cells.

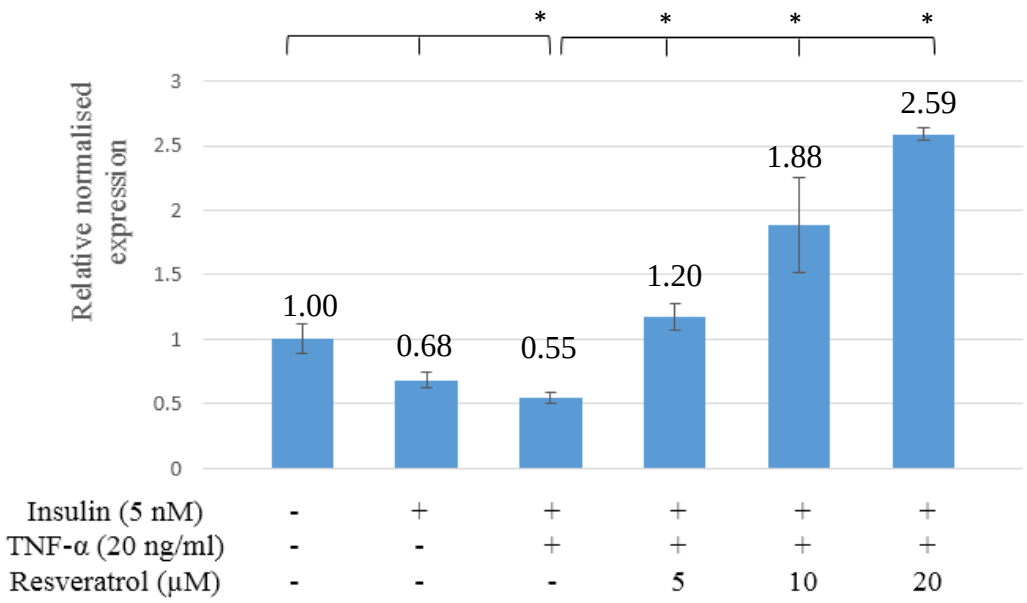


Figure 4.9: Graphical representation showing dose response of ABCA1 expression in HepG2 cells stimulated by 5 nM insulin, 20 ng/ml TNF-α and 5 μM, 10 μM and 20 μM of resveratrol

This gene expression chart of ABCA1 was analysed by the program in CFX96^{TM} Real-Time PCR Detection System. The concentration of resveratrol is indicated on X-axis while the relative normalised gene expression is represented on Y-axis. The value above each bar chart was interpreted as the fold value of ABCA1 gene expression which has been normalised to β-actin and compared with the negative control (untreated HepG2 cells). The value of relative normalised expression for untreated sample was assigned as 1.00. Error bars in each bar chart represented the standard deviation. Student's *t* test (two-sided) was used for statistical analysis. * indicates $p<0.05$ which represents statistically significant change from untreated HepG2 cells or TNF-α-treated HepG2 cells.

CHAPTER 5

DISCUSSION

5.1 Overview

There were four objectives in this study. The first objective was to identify the impact of different concentration of insulin on ABCA1 mRNA expression in HepG2 cell lines. The second objective was to discover the optimal concentration of insulin for significant changes of activity of ABCA1 expression in HepG2 cell lines. Understanding the effect of insulin and TNF-α in combination in ABCA1 expression in HepG2 cell lines was also another one of the objectives in this study. For the last objective, the expression of ABCA1 in insulin TNF-α-treated HepG2 cells treated with different antioxidants was studied by using quantitative RT-PCR.

5.2 HepG2 cells acted as a model system in this research study

Immortalised liver-derived cell line, HepG2 cells line is extensively used in toxicological and pharmacological studies. HepG2 cells line is a popular cell line used as a research tool due to its high and unlimited availability, unlike primary human hepatocyte which needs a lot of fresh human liver samples because of its limited life span (Madan, et al., 2003; Sassa, et al., 1987). HepG2 cells are highly differentiated cells and perform many functions as normal liver cells (Gerets, et al., 2009). Although they are a type of hepatoblastoma cell, bile canaliculi and Golgi apparatus are still developed as in primary human hepatocytes (Bouma, et al., 1989; Kelm, et al., 2003). Besides, they also retain the ability to synthesise

hepatic proteins such as apolipoproteins, albumin, alpha-fetoprotein, transferrin and others liver protein except for C-reactive protein (Bouma, et al., 1989). Apart from plasma protein, HepG2 cells also produce a few drug-metabolising enzymes such as cytochrome P450s and cellular transporters (Faqi, 2013). However, the expression level of these transporters and enzymes involved in biotransformation are 50-fold lower than hepatocytes *in vivo* or in primary human hepatocytes cell culture (Faqi, 2013). These differences limit the estimation when doing toxicity test for certain chemicals (Faqi, 2013). ABCA1 is also one of the transporters expressed in HepG2 cells which acts as an efflux transporter (Rodrigues, et al., 2009). Different from others transporters, ABCA1 transporter is expressed constitutively in HepG2 cells (Denis, et al., 2008).

Besides, liver-specific metabolic functions of HepG2 cells, such as cholesterol and triglyceride metabolism is still intact. According to Kohjima, et al. (2008), stimulation by unsaturated fatty acid such as α-linolenic acid and linoleic acid showed suppressive effect on the hepatic fatty acid production and fatty acid oxidation pathways. This article proved that HepG2 cells are still able to respond to external stimuli and carry out the lipid metabolism similar to the primary human hepatocytes (Kohjima, et al., 2008). In addition, HepG2 cells could act as a target for cytokine and involved in expressing various cytokines such as interferon gamma (IFN-γ), TNF-α, transforming growth factor β (TGF- β) and macrophage colony stimulating factor (M-CSF) (Stonans, et al., 1999). Some of these cytokines could act on hepatocytes itself, while others are known to regulate other cells for example, leukocyte, Kupffer cells and endothelial cells (Stonans, et al., 1999).

5.3 Total cellular RNA isolation by Tri-Reagent® LS

Tri-Reagent® LS was recommended for total cellular RNA isolation after each treatment because RNA, DNA and protein can be isolated simultaneously through single-step liquid separation. This RNA isolation method was an improved technique from Chomczynski and Sacchi (2006) and then it could be completed within one hour. Tri-Reagent® LS consists mixture of guanidine thiocyanate and phenol which is responsible in dissolving DNA, RNA and protein from homogenised tissue sample effectively. Guanidine thiocyanate helps to inhibit the activity of nucleases, RNase. Phenol and 1-Bromo-3-Chloropropane (BCP) are for separating the aqueous solution of homogenised tissue into two phases, the upper aqueous phase and the lower organic phase. BCP is responsible to recover and improve the RNA yield whereas acidic phenol helps to retain the DNA in phenol phase (Palmiter, 1974; Revest and Longstaff, 1998). The purpose of these two actions is to maximise the recovery amount of RNA.

5.4 RNA integrity and purity

In this studies, RNA integrity was assessed by subjecting the samples to 1% denaturing agarose-bleach gel. When carrying out the gel electrophoresis, RNA from each samples fractionated into its component parts, which are mRNA, tRNA and rRNA according to their molecular weight (Ream and Field, 1999). However, mRNA was not visible after the gel staining step because it only constitutes small percentage which is only about 5% of the total RNA (Ream and Field, 1999). In contrast, rRNA occupies the most, 75% in the total cellular

RNA (Ream and Field, 1999). Therefore, rRNA was a suitable target among the RNA population to be used as a tool to reveal the underlying mRNA population.

In denaturing condition, the secondary structure of RNA was disrupted in which the hydrogen bond was destroyed and RNA was exposed as single-stranded molecules so that the RNA molecules could migrate and separate properly based on their size (Masek, et al., 2005). Commercial bleach was added in the agarose gel to denature the secondary structure formed via intramolecular base pairing (Aranda, et al., 2012). This step greatly improved the mobility of the RNA, helped a better separation in electrophoresis and reduced the electrical resistance in electric field.

According to study by Aranda, et al. (2012), bleach gel was shown to be able to destroy any RNases. RNases is the major concern which could affect the integrity of RNA because it could be found anywhere especially contaminating laboratory samples. Its resistance to extreme temperature (15-80℃) and chemical insults could compromise the RNA in research sample if it was not destroyed.

Spectrophotometer measurement was also carried out to confirm the RNA was pure, preventing the result of downstream application, qRT-PCR was affected. The ratio of optical density 260 nm to 280 nm greater than 1.8 represents acceptable range, showing the RNA is pure (Manchester, 1996). Optical density at 280 nm provides the estimation information for protein while A_{230} reveals the presence of residual organic contaminants (Imbeaud, et al., 2005; Manchester,

1996). Totally pure RNA should also have value larger than 1.8 in ratio A_{260} to A_{230} (Imbeaud, et al., 2005). From the results of this experiment, all the RNA samples showed a range of optical density from 1.99 to 2.05, representing that the RNA was pure and without contaminants. Assessments on the purity and integrity of RNA were carried out to confirm the RNA was qualified to be used in subsequent step, qRT-PCR (Jose and Belen, 2012). The reason for checking the RNA quality because it is an important factor affecting the reproducibility and biological relevance of qRT-PCR (Jose and Belen, 2012).

5.5 Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

5.5.1 Principles behind qRT-PCR

Quantitative RT-PCR is an evolution or improved method from conventional PCR which allows precise quantification of desired nucleic acid (Higuchi, et al., 1992). Alteration of gene expression level, ABCA1 in this experiment under stimuli by insulin, TNF-α, lauric acid and resveratrol was measured by qRT-PCR because it only needed relatively small amount of RNA samples to produce rapid and accurate data.

To detect the product of PCR, nonspecific fluorescent DNA-binding dyes, SYBR green was used. They are easier to optimise during qRT-PCR and cheaper compared to strand-specific probes (Fraga, et al., 2008). Upon binding to double-stranded DNA, their fluorescence intensity spike 20- to 100- fold. However, the DNA fragments obtained from PCR process has to be single and specific when using SYBR green because nonspecific DNA fragment will lead to inaccurate

increase of fluorescence intensity (Bookout and Mangelsdor, 2003). Therefore, PCR reactions should be optimised to eliminate other unspecific amplicon and make sure only the target amplicon is present (Fraga, et al., 2008). Besides, other method such as melting point analysis can be used to differentiate either specific product or the contamination by other unspecific PCR products (Fraga, et al., 2008).

5.5.2 Amplification plots from qRT-PCT

Amplification plots were shown in Figure 4.5. In the plots, three phases, exponential phase, linear phase and plateau phase could be observed. If the fluorescence signal reached above the threshold or background, then it is considered as reliable signal. The cycle which exceeded the threshold is known as cycle threshold (C_q). Threshold setting step is a critical step because too low or too high of the threshold value may produce unreliable data. The quantity of initial templates was reflected by the quantity of PCR products in exponential phase. To make the PCR amplification achieved approximate 100%, certain conditions such as primer characteristics, template purity and PCR products should be in optimal state (Fraga, et al., 2008).

5.5.3 Melt curve analysis

Melting curves in Figure 4.6 were plotted in first negative differential of the fluorescence intensity against the temperature in X-axis. At low temperature, the amplicons were in double-stranded form (Fraga, et al., 2008). SYBR Green dye bound to these amplicons, emitting strong fluorescence signal. The PCR products were then eventually denatured by the gradually increasing temperature

and the SYBR Green dye dissociated from the amplicons, resulting decreasing in fluorescence signal (Ririe, et al., 1997). The fluorescence intensity was measured continuously until the melting point, T_m of dsDNA was detected before the fluorescence signal decreased rapidly over a short temperature range (Ririe, et al., 1997). Different PCR products has different melting point and therefore different peaks could be generated for respective amplicons (Fraga, et al., 2008). Usually, PCR artifacts such as primer-dimer and misprimed products have lower melting point than the desired PCR products (Fraga, et al., 2008; Wittwer, et al., 1997). Therefore, the artifact products can be distinguished using melting point analysis. The PCR thermal cycler was programmed at the appropriate temperature, above the melting point of PCR artifacts but below the melting point of target PCR products so that only the fluorescence measurement of target products was included in analysis (Arya, et al., 2005). Based on Figure 4.6, the melting curve only presented one sharp peak for each gene, ABCA1 and β-actin. One sharp peak indicates that there was no contamination or primer-dimers formation and only desired targets, ABCA1 and β-actin were amplified.

5.5.4 Relative quantification

In this study, relative quantification was used. Housekeeping genes, β-actin was used as an internal standard to normalise the changes in the ABCA1 gene expression (Mori, et al., 2008). Besides β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is also extensively used as constitutive housekeeping genes in qRT-PCR (Mori, et al., 2008). They are expressed constitutively in every cell among different tissues of an organism, different level of development and different experimental stimuli and one assumption were made where they

should not be affected by any treatments (Glare, et al., 2002). However, GADPH was not suitable to be used in this study due to its excessive expression in the cancer cell lines (Zhong and Simons, 1999). Nevertheless, β-actin gene exhibits stable gene expression in a study, the inflammation mechanism of rats was induced (Jiang, et al., 2009). According to Chew, et al. (2007), β-actin was proven, a better option because it is expressed constantly in human HepG2 cells stimulated by TNF-σ.

5.6 Effect of insulin on ABCA1 expression

Insulin displayed a suppressive pattern on ABCA1 gene expression in a dose dependent manner as shown in Figure 4.7. A decreasing trend could be observed from Figure 4.7. ABCA1 was significantly suppressed to 0.33-fold by 5 nM of insulin and hence it was chosen as the optimal dose for all subsequent treatments. Although 100 nM showed the highest suppressive effect 0.26-fold of ABCA1 suppression, but the lower dose of insulin was used in this study because its suppression was significant in that concentration of insulin.

In the presence of insulin, the HDL biogenesis was impaired (Nonomura, et al., 2011). Insulin blocked the activity of ABCA1 by modulating two different mechanisms. Firstly, insulin has been shown to intensify the degradation rate of ABCA1 (Nonomura, et al., 2011). In the second mechanism, phosphorylation of Tyr1206 of ABCA1 by insulin further decreased the cholesterol efflux due to diminished of specific activity in phosphorylated ABCA1(Nonomura, et al., 2011). Therefore, the results obtained in this study are reflective of the results from these studies.

Binding of insulin to PI3K/Akt was shown to influence the lipid metabolism directly (Krycer, et al., 2010). Insulin, is an important catalyst in activating SREBP-1c to become mature and transcriptionally active form through mTORC1, which is a downstream target of Akt and acted as suppressor to the cholesterol efflux event (Li, et al., 2010). Additionally, insulin signaling pathway also requires SREBP-2 activation which was also directly activated by Akt (Miao, et al., 2014). SREBP-1c and SREBP-2 are isoforms of the sterol regulatory element-binding proteins (SREBPs) which facilitates in lipid homeostasis (Brown and Goldstein, 1997). SREBP-1c takes place in triggering the pathways of fatty acid triglyceride and phospholipid pathways whereas induction of cholesterol synthesis and uptake involves SREBP-2 isoform (Brown and Goldstein, 1997; Norton, et al., 1998). SREBP-1c is capable of causing hypertriglycemia metabolic syndrome due to the induction signal by insulin (Copeland, et al., 2001; Kim, et al., 1998). Moreover, SREBP-2 is well known for its suppressive effect on ABCA1 transcription in vascular endothelial cells. Therefore, it is hypothesized that insulin reduces ABCA1 expression via the regulatory factor SREBP-2 in HepG2 cells. Figure 5.1 exhibits several pathways that are manipulated by insulin to regulate the lipid metabolism in cells.

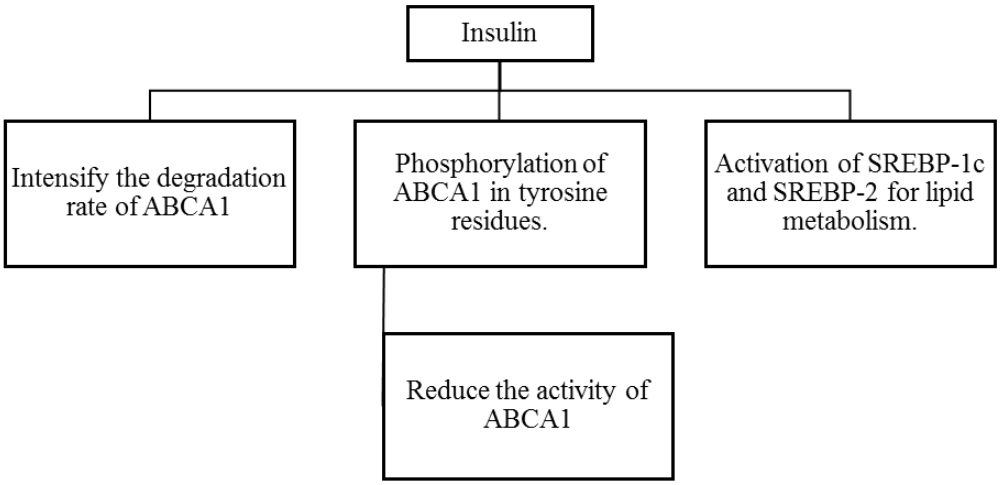


Figure 5.1: Pathways that insulin used to regulate the lipid metabolism in cells

Insulin interrupts and impairs the function of ABCA1 through the three patterns above. Low level of ABCA1 in the cells is due to high breakdown rate of ABCA1 by insulin. Besides, tyrosine phosphorylation of ABCA1 contributes to the low efficacy of ABCA1 activity. In lipid metabolism, SREBP-1c and SREBP-2 are responsible for the buildup of bad cholesterol in the cells.

5.7 Effect of TNF-α and insulin on ABCA1 expression

In Figure 4.8 and Figure 4.9, TNF-α worked synergistically with insulin and further decreased the ABCA1 mRNA expression. TNF-α is always associated with insulin resistance in adipose tissue, muscle and hepatocytes (Plomgaard, et al., 2005; Porter, et al., 2002; Xu, et al., 2008). Researchers have discovered that overexpression and presence of TNF-α i*n vivo* and *in vitro* contributed to reduction of insulin sensitivity in cells (Hotamisligil, et al., 1996; Shibasaki, et al., 2003). TNF-α acts as a negative regulator in insulin signalling pathway (Rocha, et al., 2014). Inflammation activity triggered by TNF-α contributed to the activation of mitogen-activated protein kinases (MAPKs), activator protein-1 and NF-κB (Jin, et al., 2011). MAPKs is responsible in increasing the serine kinase for serine phosphorylation of insulin receptor substrate-1 (IRS-1) and these phosphorylated IRS-1 are then subjected to ubiquitination (Boura-Halfon and Zick, 2008; Gual, et al., 2005; Sun, et al., 1999). Ubiquitinated IRS-1 negatively regulates the insulin effect (Sun, et al., 1999).

In normal condition, the binding of insulin to insulin receptor enhances tyrosine phosphorylation of IRS-1 and hence activates insulin downstream signalling pathway such as phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt/PKB) (Khan and Pessin, 2002). However, NF-κB triggered by TNF-α stops these activities by upregulating the gene expression of protein tyrosine phosphatase-1B (Zabolotny, et al., 2008). This molecule impairs the insulin signal transduction pathway by inhibiting the tyrosine phosphorylation in IRS-1 (Zabolotny, et al., 2008). Furthermore, the presence of Akt is important for the phosphorylation of Forkhead transcription factors, FoxO1 which plays pivotal

role in glucose and lipid metabolism in the liver (Fan, et al., 2009). However, reduced Akt decreases the Akt-dependent phosphorylation of FoxO1 (Fan, et al., 2009). Thus, the unphosphorylated FoxO1 cannot be sequestered from nucleus to cytoplasm (Tzivion, et al., 2011). FoxO1 will then bind to peroxisome proliferator-activated receptor-γ (PPARγ) promoter and prevented PPARγ gene expression (Armoni, et al., 2006). Previous studies have shown that PPARγ is a vital nuclear receptor in cholesterol metabolism of macrophage cells (Toh, et al., 2011). However, suppression of PPARγ gene by FoxO1 also reduces the LXRα gene expression (Chawla, et al., 2001). PPARγ-LXRα-ABCA1 gene expression pathway is then restricted and hence cholesterol efflux in macrophages will not be carried out due to lack of ABCA1 (Chawla, et al., 2001). Figure 5.2 summarizes the putative pathways of TNF-α in ABCA1 gene expression.

According to Zhao and Dong (2008), 20 ng/ml of TNF-α was sufficient to show suppressive effect on ABCA1 gene expression in adipocytes. Therefore, this specific dose was used in entire experiment. Although TNF-α blocks the insulin signalling pathway and induces insulin resistance in HepG2 cells, it was still able to inhibit ABCA1 gene expression significantly without the assistance of insulin as shown in Figure 4.8 and Figure 4.9. This proves that TNF-α has suppressive effect on HepG2 cells lines. As described earlier in literature review, TNF-α impaired ABCA1 expression in THP-1 macrophage-derived foam cells and human intestinal cell line and contributed to accumulation of cholesterol in the cells (Field, et al., 2010; Mei, et al., 2007). Similar effects was also shown in HepG2 cells in which TNF-α deactivated ABCA1 expression to impair the formation of nascent HDL. In addition, ABCA1 turnover rate may be increased

with the presence of TNF-α and hence cholesterol efflux was made impossible as shown by Wang and Oram (2001).

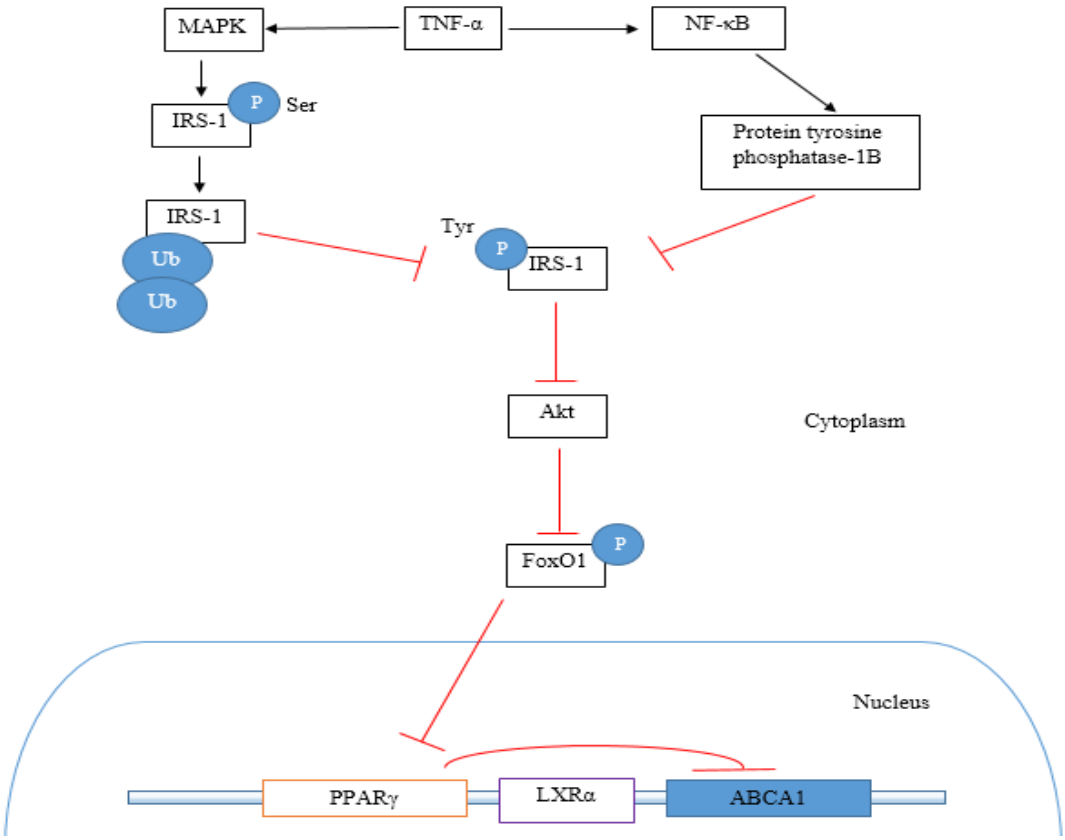


Figure 5.2: Putative model showing the proposed pathway taken by TNF-α to exert its negative effects on the ABCA1 gene expression

TNF-α inhibits the tyrosine phosphorylation in IRS-1 through MAPK and NF-κB pathways. IRS-1 is pivotal in regulating the insulin induced Akt pathway. Therefore, dysfunctional IRS-1 is unable to phosphorylate FoxO1. Unphosphorylated FoxO1 remains in the nucleus and is bound to PPARγ to inhibit PPARγ gene expression. Thus, the entire PPARγ-LXRα-ABCA1 gene expression pathway will cease to function.

5.8 Effect of lauric acid on ABCA1 expression

Augmentation of ABCA1 expression by stimulation of lauric acid is presented in Figure 4.8. The suppression effect of TNF-α and 5 nM of insulin could be reversed to the untreated condition by the addition of lauric acid. In 5 μM, 10 μM and 20 μM of lauric acid, the ABCA1 expression was increased to 1.05-fold, 1.22-fold and 1.72-fold respectively, considered as a significant increment.

Lauric acid has been shown to act as an agonist to LXRα which in turn upregulated the ABCA1 gene expression (Jun, et al., 2013; Ozasa, et al., 2011). SREBP-2 is responsible for binding to the E-box situated in the ABCA1 promoter was then reduced by the activated LXR. Inability of SREBP-2 to bind to ABCA1 promoter and upregulation of LXR proved to produce a synergistic effect in stimulating ABCA1 expression (Zhou, et al., 2009).

Besides, lauric acid comes with anti-inflammatory ability to inhibit the expression of transcription factor, NF-κB (Kim, et al., 2008; Pengseng, et al., 2013). Switching off NF-κB, a pro-inflammatory transcription factor leads to deactivation of immune response and inflammatory response could be a way to lauric acid's inflammatory role (Collins, et al., 1995; Zhu, et al., 2013). This is because NF-κB plays a pivotal role in controlling the synthesis of broad spectrum of cytokines involved in inflammation and immunity (Collins, et al., 1995; Zhu, et al., 2013). Inhibition of NF-κB by lauric acid would cause TNF-α to lose its suppressive effect on ABCA1 gene expression (Wang, et al., 2010). In addition, according to Chen, et al. (2006), NF-κB is a key factor which regulates ABCA1 basal expression. Hence, the inhibition of ABCA1 in insulin

treatment condition could be reversed by lauric acid by its action NF-κB (Chen, et al., 2006).

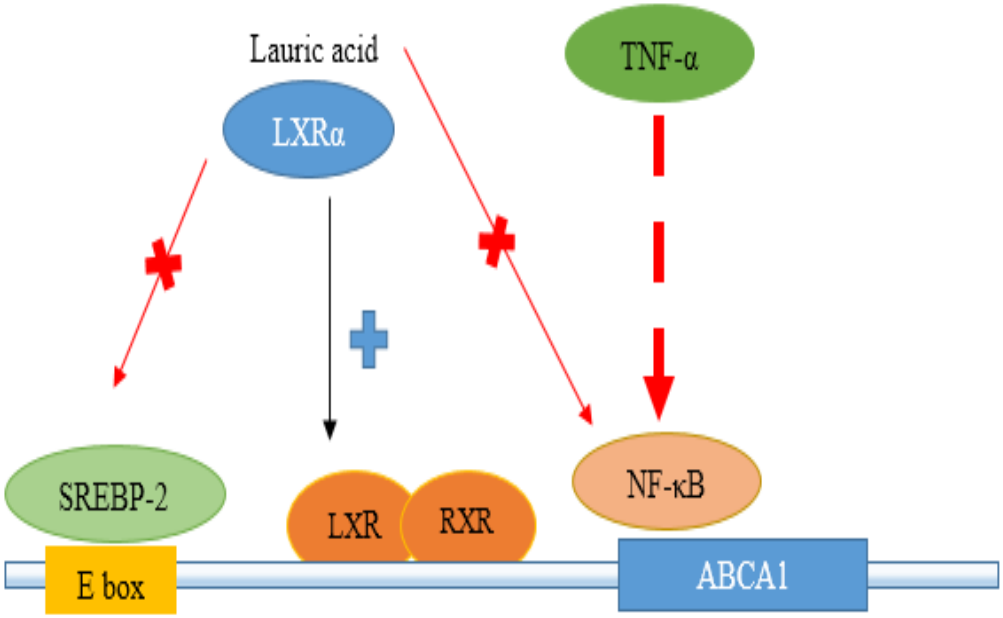


Figure 5.3: Hypothetical scheme showing the mechanism of lauric acid in promoting ABCA1 gene expression through different pathways

Binding of lauric acid to LXRα activates ABCA1 gene expression. Besides, SREBP-2 is blocked from acting on E box by the activated LXR and hence SREBP-2 cannot inhibit activity of ABCA1 gene expression. In addition, anti-inflammatory properties of lauric acid tends to target on and shut down NF-κB pathway. The suppressive effect of TNF-α on ABCA1 is cancelled due to dysregulation of NF-κB. These activity results in recovery of ABCA1 activity.

5.9 Effect of resveratrol on ABCA1 expression

In 5 μM, 10 μM and 20 μM of resveratrol, ABCA1 expression was increased significantly which were 1.20-fold, 1.88-fold and 2.59-fold respectively. Similar to lauric acid, resveratrol could reverse the inhibitory effect of insulin and TNF-α. From this study, resveratrol has greater stimulatory effect as compared to lauric acid as observed from Figure 4.9.

Resveratrol is a natural product found in red grapes and nuts, and it has been proven to have ability to suppress the expression of NF-κB subunit (Zhu, et al., 2011). Resveratrol could attenuate TNF-α induced acetylation, phosphorylation and nuclear translocation of NF-κB subunit in order to prevent NF-κB induced pro-inflammatory molecules production (Bishayee, et al., 2010; Kumar and Sharma, 2010). In addition, IKK activity was cancelled by resveratrol and it was unable to phosphorylate IκB (Holmes-McNary and Baldwin, 2000). Hence, IκB arrested the NF-κB in cytoplasm and ameliorated the inflammation response. Besides, resveratrol was shown to be able to alleviate TNF-α induced inflammation via Sirtuin 1 (Sirt1), an anti-inflammatory regulator (Zhu, et al., 2011). By acting on Sirt1, pro-inflammatory mediators such as interleukin-1beta (IL-1β), inducible nitric oxide synthase (iNOS) and interleukin-6 (IL-6) induced by TNF-α were suppressed (Csaki, et al., 2008). Therefore, Sirt1 is suggested as an efficient target for resveratrol to reduce the suppression effect of insulin resistant state by TNF-α.

According to Chang, et al. (2012), resveratrol also induces LXRα, a key transcriptional factor for ABCA1 in nuclear level of macrophages (Sevov, et al.,

2006) and thus upregulates LXRα-dependent upregulation of ABCA1 (Chang, et al., 2012). Resveratrol is then able to indirectly prevent the lipid accumulation in the cells through the effective ABCA1 and helping in cholesterol efflux. Therefore, the similar outcome, elevation of ABCA1 expression is expected to happen in HepG2 cells. Figure 5.4 shows the schematic diagram of putative model used by resveratrol in diminishing inhibitory effect of TNF-α on ABCA1.

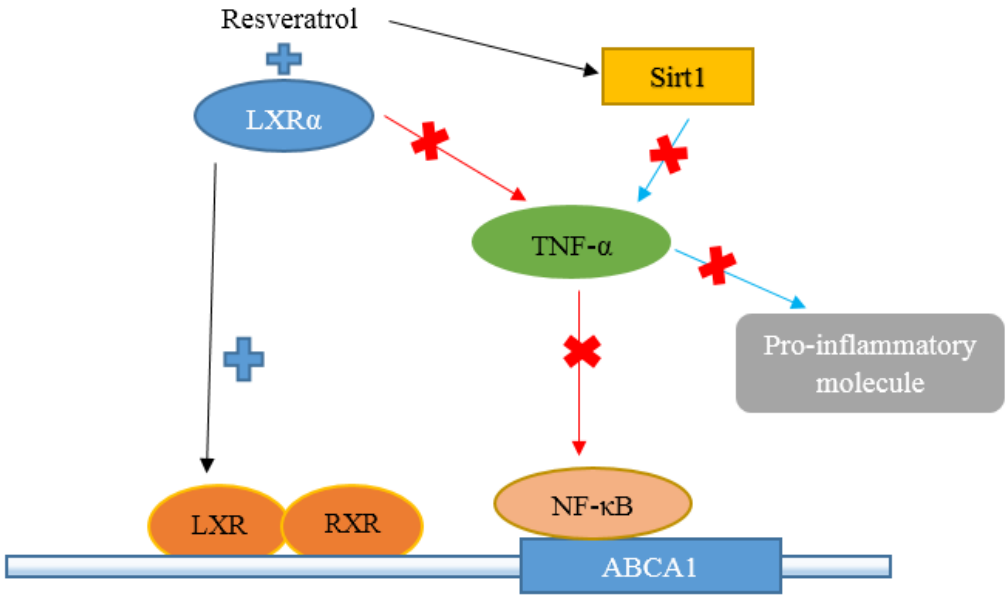


Figure 5.4: This diagram shows putative model manipulated by resveratrol in attenuating inhibitory action of TNF-α on ABCA1

Resveratrol enhances the activity of LXRα and hence the ABCA1 gene expression is upregulated. Activation of LXRα also helps to suppress the activity of TNF-α and indirectly switch off the NF-κB inhibition effect on ABCA1. Moreover, resveratrol activates Sirt1 which assists to suppress the inhibitory action exerted by TNF-α and prevent the production of pro-inflammatory molecule.

5.10 Future studies

In this experiment, only TNF-α was used to evaluate ABCA1 gene expression. Different types of pro-inflammatory molecules can be used in the future to study ABCA1 expression in HepG2 cells and to evaluate the viability of the cells. This idea is suggested in order to discover whether other cytokines have similar inhibitory effect as TNF-α in the presence of insulin.

The actual and detailed mechanism manipulated by insulin, lauric acid and resveratrol in regulating effect of TNF-α on ABCA1 gene expression in HepG2 cells can be investigated through knockdown assay on a few of the key regulators such as Akt, PPARγ, FoxO1 and Sirt1. Moreover, ABCA1 protein level when HepG2 cells is exposed to lauric acid and resveratrol can be investigated to verify if the effects of transcription level is translated to the protein level.

Moreover, various type of fatty acid besides lauric acid, with different carbon atom number can be used to detect their effects on gene expression of ABCA1. Besides, both unsaturated fatty acids and saturated fatty acids can also be used to gain the information about how the structural difference of fatty acid can affect the ABCA1 expression. In addition, antioxidants such as kaempferol can be utilised to compare with lauric acid and resveratrol in order to determine which antioxidant has the highest upregulatory effect on expression of ABCA1 gene.

CHAPTER 6

CONCLUSIONS

This study was successfully conducted and the objectives were fulfilled. The effects of insulin, TNF-α and antioxidants on ABCA1 mRNA expression were fully investigated and understood. Due to its sensitivity, qRT-PCR was used in this study because it can provide precise quantification of desired nucleic acid. β-actin, an internal control gene was used to normalise ABCA1 mRNA expression.

With the increasing dose of insulin, ABCA1 gene expression was suppressed. All the suppressive effects by insulin were statistically significant in a dose dependent manner. ABCA1 gene expression was downregulated by insulin in a dose-dependent manner. With the help from 20 ng/ml of TNF-α, ABCA1 gene expression was further suppressed. This indicates that combination of TNF-α and insulin had the similar inhibitory effect as the treatment with insulin only.

Lauric acid and resveratrol showed the same ability in which they could cancel the suppressive effect exerted by TNF-α and insulin and upregulate ABCA1 gene expression. Overall, these two antioxidants increased the expression of ABCA1 gene when the cells were treated with increasing concentrations of the antioxidants and all the increments were statistically significant.

As a conclusion, insulin and TNF-α repressed ABCA1 gene expression in insulin resistant state. However, lauric acid and resveratrol abrogated the inhibitory effects by insulin and TNF-α and restored the ABCA1 gene expression activity, indicating their antioxidative properties, and the positive outlook to relieve lipid transport in insulin resistant state.

CHAPTER 7

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CHAPTER 8

APPENDICES

Appendix A

96-wells plate diagram for qRT-PCR

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Unt**	**Unt**	**Unt**	**1 nM insulin**	**1 nM insulin**	**1 nM insulin**	**5 nM insulin**	**5 nM insulin**				
B	**5 nM insulin**	**10 nM insulin**	**10 nM insulin**	**10 nM insulin**	**50 nM insulin**	**50 nM insulin**	**50 nM insulin**	**100 nM insulin**				
C	**100 nM insulin**	**100 nM insulin**	**Unt**	**Unt**	**Unt**	**1 nM insulin**	**1 nM insulin**	**1 nM insulin**				
D	**5 nM insulin**	**5 nM insulin**	**5 nM insulin**	**10 nM insulin**	**10 nM insulin**	**10 nM insulin**	**50 nM insulin**	**50 nM insulin**				
E	**50 nM insulin**	**100 nM insulin**	**100 nM insulin**	**100 nM insulin**								
F												
G												
H												

This represents ABCA1

This represents β-actin

Appendix B

96-wells plate diagram for qRT-PCR

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Unt**	**Unt**	**Unt**	**Ins.**	**Ins.**	**Ins.**	**Ins.+ TNF**	**Ins.+ TNF**				
B	**Ins.+ TNF**	**Ins.+ TNF+ 5 µM LA**	**Ins.+ TNF+ 5 µM LA**	**Ins.+ TNF+ 5 µM LA**	**Ins. + TNF+ 10 µM LA**	**Ins. + TNF+ 10 µM LA**	**Ins. + TNF+ 10 µM LA**	**Ins. + TNF+ 20 µM LA**				
C	**Ins. + TNF+ 20 µM LA**	**Ins. + TNF+ 20 µM LA**	**Unt**	**Unt**	**Unt**	**Ins.**	**Ins.**	**Ins.**				
D	**Ins.+ TNF**	**Ins.+ TNF**	**Ins.+ TNF**	**Ins.+ TNF+ 5 µM LA**	**Ins. + TNF+ 5 µM LA**	**Ins. + TNF+ 5 µM LA**	**Ins. + TNF+ 10 µM LA**	**Ins. + TNF+ 10 µM LA**				
E	**Ins. + TNF+ 10 µM LA**	**Ins. + TNF+ 20 µM LA**	**Ins. + TNF+ 20 µM LA**	**Ins. + TNF+ 20 µM LA**								
F												
G												
H												

5 nM of Insulin in short form “Ins.”
20 ng/ml of TNF-α is represented by “TNF”
Lauric acid in short form “LA”

This represents ABCA1

This represents β-actin

Appendix C

96-wells plate diagram for qRT-PCR

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Unt**	**Unt**	**Unt**	**Ins.**	**Ins.**	**Ins.**	**Ins.+ TNF**	**Ins.+ TNF**				
B	**Ins.+ TNF**	**Ins.+ TNF+ 5 µM RE**	**Ins.+ TNF+ 5 µM RE**	**Ins.+ TNF+ 5 µM RE**	**Ins. + TNF+ 10 µM RE**	**Ins. + TNF+ 10 µM RE**	**Ins. + TNF+ 10 µM RE**	**Ins. + TNF+ 20 µM RE**				
C	**Ins. + TNF+ 20 µM RE**	**Ins. + TNF+ 20 µM RE**	**Unt**	**Unt**	**Unt**	**Ins.**	**Ins.**	**Ins.**				
D	**Ins.+ TNF**	**Ins.+ TNF**	**Ins.+ TNF**	**Ins.+ TNF+ 5 µM RE**	**Ins. + TNF+ 5 µM RE**	**Ins. + TNF+ 5 µM RE**	**Ins. + TNF+ 10 µM RE**	**Ins. + TNF+ 10 µM RE**				
E	**Ins. + TNF+ 10 µM RE**	**Ins. + TNF+ 20 µM RE**	**Ins. + TNF+ 20 µM RE**	**Ins. + TNF+ 20 µM RE**								
F												
G												
H												

5 nM of Insulin in short form “Ins.”
20 ng/ml of TNF-α is represented by “TNF”
Resveratrol in short form “RE”

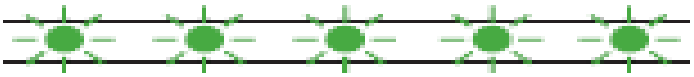
This represents ABCA1

This represents β-actin

Appendix D

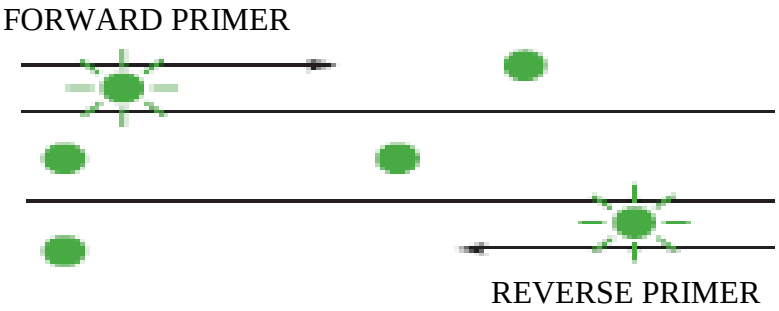
SYBR Green dye assay

1. **Reaction setup:** The SYBR Green dye fluoresces when bound to double-stranded DNA.



2. **Denaturation:** When the DNA is denatured, the SYBR Green dye is released and the fluorescence is drastically reduced.

3. **Polymerisation:** During extension, primers anneal and PCR product is generated.



4. **Polymerisation completed:** When polymerisation is complete, SYBR Green dye binds to the double-stranded product, resulting in a net increase in fluorescence.