THE EFFECTS OF ANTIOXIDANTS KAEMPFEROL AND ASCORBIC ACID ON LIVER X RECEPTOR ALPHA (LXR-α) IN TNF-α STIMULATED HUMAN HEPATOCARCINOMA HEPG2 CELLS

By

YU YEN EE

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ABSTRACT

THE EFFECTS OF ANTIOXIDANTS KAEMPFEROL AND ASCORBIC ACID ON LIVER X RECEPTOR ALPHA (LXR-α) IN TNF-α STIMULATED HUMAN HEPATOCARCINOMA HEPG2 CELLS

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Liver X Receptor alpha (LXR-α) plays an essential roles in cholesterol metabolism, bile acids and lipid synthesis. In this study, the effects of kaempferol and ascorbic acid on LXR-α mRNA expression in TNF-α stimulated HepG2 cells was investigated. After the cells have reached 60-70% confluency, they were treated with 20 ng/ml of TNF-α for 22 hours prior to the stimulation with different concentrations of kaempferol (1 μM, 5 μM, 10 μM and 20 μM) or ascorbic acid (15 μM, 150 μM and 1500 μM) for another 22 hours. Total RNA was isolated using TRI Reagent ®LS (Molecular Research Centre). Spectrophotometric measurement showed that A_{260} /A_{280} value of the extracted RNA was in the range of 2.0, whereas the fluoresencent ratio of 28S rRNA and 18S rRNA was 2:1 in 1% denaturing agarose-formaldehyde gel. This indicated that extracted RNA was intact and pure. Real Time RT-PCR was then carried out to determine the mRNA expression of LXR-α after their respective treatments. Results showed that kaempferol was able to relieve the inflammatory action of TNF-α. The highest dose of kaempferol (20 μM) almost abrogated the down-regulation of LXR-α mRNA expression by TNF-α

to 0.927-fold of control sample. Twenty μM of kaempferol alone did not produce any significant effect on LXR-α mRNA expression. In contrast, ascorbic acid was unable to counteract the action of TNF-α. Contrary to kaempferol, the highest dose of ascorbic acid (1500 μM) suppressed the expression of LXR-α mRNA in TNF-α stimulated HepG2 cells to 0.571-fold compared to untreated sample. In the presence of 1500 μM of ascorbic acid alone, LXR-α mRNA expression was significantly suppressed to 0.320-fold. In conclusion, this study successfully profiled that kaempferol, but not ascorbic acid, was able to alleviate the TNF-α down-regulatory effects on LXR-α mRNA in HepG2 cells.

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Lastly, I would like to thank my family for their emotional support and concern which helped me to complete my project.

DECLARATION

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

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YU YEN EE

APPROVAL SHEET

This project report entitled "**THE EFFECTS OF ANTIOXIDANTS KAEMPFEROL AND ASCORBIC ACID ON LIVER X RECEPTOR ALPHA (LXR-α) IN TNF-α STIMULATED HUMAN HEPATOCARCINOMA HEPG2 CELLS"** was prepared by YU YEN EE 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:

_____

(DR. CHEW CHOY HOONG) Date:……..

Assistant Professor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **YU YEN EE** (ID No: **08ADB04534**) has completed this final year project entitled "**THE EFFECTS OF ANTIOXIDANTS KAEMPFEROL AND ASCORBIC ACID ON LIVER X RECEPTOR ALPHA (LXR-α) IN TNF-α STIMULATED HUMAN HEPATOCARCINOMA HEPG2 CELLS**" supervised by Dr. Chew Choy Hoong (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to my supervisors 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.

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LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260nm wavelength
A_{280}	Absorbance at 280nm wavelength
ABC	ATP-binding cassette
ACAT	Acyl-CoA: cholesterol O-aclytransferase
ACC	Acetyl-CoA carboxylase
ACHN	Human renal carcinoma cell line
AF	Activation function
Apo	Apolipoprotein
APR	Acute phase response
ATCC	American Type Culture Collection
β-actin	Beta-actin
BCP	1-bromo-3-chloropropane
CA	Co-activator
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CETP	Cholesterol ester transfer protein
ChREBP	Carbohydrate response element binding protein
CMT	Charcot-Marie-Tooth
CO_2	Carbon dioxide
COX	Cyclooxygenase
CR	Co-repressor
CRP	C-reactive protein

C_T	Cycle threshold
CYP7A1	Cholesterol 7α-hydroxylase
DBD	DNA binding domain
DHAA	Dehydroascorbic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DR	Direct repeats
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
EtBr	Ethidium bromide
FAS	Fatty acid synthase
FBS	Foetal bovine serum
G6Pase	Glucose-6-phosphatase
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanidine and cytosine
G-CSF	Granulocyte colony-stimulating factor
GLUT	Glucose transporter
GSH	Glutathione
HDL	High density lipoprotein
HNF	Hepatocyte nuclear factor
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
ICAM	Intercellular adhesion molecule
IKK	IκB kinase

IL	Interleukin
iNOS	Inducible nitric oxide synthase
LBD	Ligand binding domain
LCAT	Lecithin: cholesterolacyl transferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LXR	Liver X receptor
LXRE	Liver X receptor response element
LXR-α	Liver X receptor alpha
LXR-β	Liver X receptor beta
MCF-7	Human breast adenocarcinoma cell line
MCP	Monocyte chemoattractant proteins
M-CSF	Macrophage colony-stimulating factor
MEM	Minimum essential medium
MMP	Matrix metalloproteinases
MOPS	3-[N-mopholino] propanesulphonic acid
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NaOH	Sodium hydroxide
N-CoR	Nuclear receptor co-repressor
NF-κB	Nuclear factor-kappaB
NPC	Niemann-Pick C
NPC1L1	Niemann-Pick 1 Like 1
NR	Nuclear receptor

O_2^-	Anion radical superoxide
OD	Optical density
PBS	Phosphate-buffered saline
PEPCK	Phosphoenolpyruvate carboxykinase
PGES	Prostaglandin E synthase
PLTP	Phospholipids transfer protein
PPAR	Peroxisome proliferator-activated receptor
PPARGC1	PPAR gamma co-activator 1 alpha
PR	Progesterone receptor
RCT	Reverse cholesterol transport
RFU	Relative fluorescence units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
RXR	Retinoid X receptor
S	Svedberg unit
SAP	Serum amyloid P
SCD	Steroyl-CoA desaturase
SHP	Small heterodimer partner
SMRT	Silent mediator of retinoid acid receptor and thyroid receptor
SR-B1	Scavenger receptor type B1
SRE	Sterol response element
SREBP-1c	Sterol regulatory element-binding protein-1c
sTNF-α	Soluble tumour necrosis factor alpha

SVCT	Sodium dependent vitamin C transporter
TACE	Tumour necrosis factor alpha converting enzyme
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TG	Triglyceride
TLR	Toll-like receptors
T_m	Melting temperature
tmTNF-α	Transmembrane tumour necrosis factor alpha
TNFR	Tumour necrosis factor alpha receptor
TNF-α	Tumour necrosis factor alpha
TRRAP	Transformation/ transcription domain-associated protein
UGT1A3	Uridine diphosphate glucuronosyl transferase 1A3

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CHAPTER 1

INTRODUCTION

Liver X Receptors (LXRs) are nuclear transcription factors involved in cholesterol, bile acids, fatty acids and lipid metabolism (Hashimoto, Matsumoto, Yamada, Satoh & Mori, 2007; Baranowski, 2008). Studies have also showed that LXRs modulate carbohydrate metabolism and inflammation process (Zelcer & Tontonoz, 2006; Jamroz-Wiśniewska, Wójcicka, Horoszewicz & Bełtowski, 2007). LXRs exist in two isoforms, which are LXR-α and LXR-β. LXR-α is expressed abundantly in metabolically active tissues like liver, kidney and adipose tissue, whereas LXR-β is ubiquitously found. Both LXRs are activated by oxysterols (Zelcer & Tontonoz, 2006). Upon ligand binding, they form obligate heterodimer with Retinoid X Receptor (RXR) and bind to LXR response element (LXRE) to activate gene transcription.

TNF-α is a multifunctional cytokine synthesised by immune cells like monocytes and macrophages. It is involved in various cellular processes and diseases especially inflammation and cancers. Low level of TNF-α can stimulate our immune system but prolonged presence of TNF-α in high level can cause deleterious effects to our body. TNF-α has been shown to interfere with lipid homeostasis and its signalling is suggested to be proatherogenic (Gerbod-Giannone & et al., 2006; Tacer, Kuzman, Seliskar, Pompon & Rozman, 2007).

The presence of TNF-α signifies inflammation and chronic inflammation which is undesirable as it can disrupt cellular homeostasis. Kaempferol is a polyphenol flavonoid ubiquitously found in vascular plants, while ascorbic acid is a water-soluble micronutrient available in fruits and vegetables. Both are well-known antioxidants which can be found easily in our food. Besides acting as antioxidants, they are also anti-inflammatory agents.

There was study showing that kaempferol was able to suppress pro-inflammatory cytokine, interleukin-1β-induced MUC5AC gene expression in inflammation (Kwon et al., 2009). As for ascorbic acid, it was able to inhibit the action of TNF-α through inhibition of nuclear factor-kappaB (NF-*κ*B) signalling pathway. Ascorbic acid may also involved in the maintenance of proper lipoprotein metabolism due to its anti-oxidative property (Arai, Uchida & Nakamura, 2005). Owing to the evidences above, it is therefore hypothesised that both kaempferol and ascorbic acid can relieve the inflammatory effects introduced by TNF-α.

Thus, the objectives of this study are:

i. To investigate and quantify the effect of kaempferol on LXR-α mRNA expression in TNF-α stimulated HepG2 cell line using Real Time RT-PCR,

ii. To investigate and quantify the effect of ascorbic acid on LXR-α mRNA expression in TNF-α stimulated HepG2 cell line using Real Time RT-PCR, and

iii. To analyse the roles of kaempferol and ascorbic acid in hepatic LXR-α regulation.

CHAPTER 2

LITERATURE REVIEW

2.1 Nuclear Receptor Superfamily

Nuclear receptors (NRs) are a group of ligand-activated transcription factors that involved in the regulation of a wide range of processes such as reproduction, development and metabolism of endobiotics and xenobiotics (Zollner, Wagner & Trauner, 2010). They are the regulators of cell life and death as aberrant function of NRs can lead to diseases. A special property of NRs that make them different from other transcription factors is that they can specifically bind to small hydrophobic molecules (Germain, Altucci, Bourguet, Rochette-Egly & Gronemeyer, 2003).

Human genome sequencing revealed 48 NRs (Alaynick, 2008; Krasowskia, Ni, Hagey & Ekins, 2010). According to Nuclear Receptors Nomenclature Committee (1999), there are seven sub-families of NRs (NR0-6). Among the seven sub-families, NR0 is a special group consisting of NRs lacking either DNA binding domain (DBD) or ligand binding domain (LBD) (Robinson-Rechavi, Garcia & Laudet, 2003). All the NRs are named in the form of NR*xyz*, whereby *x* represents sub-family, *y* and *z* represents the group and the gene, respectively (Robinson-Rechavi et al., 2003).

Generally, NRs can be divided into two main classes. The first class comprised of NRs with known physiological ligand. Here, it is subdivided into endocrine

receptors and adopted orphan receptors. Endocrine receptors are exemplified by estrogen receptor (ER) and progesterone receptor (PR). Their ligands are high-affinity hormones. As for adopted orphan receptors, they interact with low-affinity dietary molecules such as bile acids and fatty acids. Examples of adopted orphan receptors are retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR). The second class of NR is known as orphan receptors as their physiological ligands are yet to be identified. Small heterodimer partner (SHP) and hepatocyte nuclear factor-4 (HNF-4) are some of the examples (Alaynick, 2008).

2.2 Liver X Receptors (LXRs)

LXRs are member of NR subfamily 1 group H, with LXR-α being the third gene (NR1H3) and LXR-β being the second gene (NR1H2) in that group. LXRs were discovered in 1995 (Oosterveer, Grefhorst, Groen, Kuipers, 2010). They were initially isolated from human liver complementary deoxyribonucleic acid (cDNA) library as orphan receptor. Soon after the physiological ligand was discovered, they are grouped under adopted orphan receptors. LXRs have been known to be the key regulator in cholesterol and carbohydrate metabolism (Reschly et al., 2008). Despite these beneficial health effects, LXRs activation was also demonstrated to be involved in lipogenesis which in turn, resulted in liver steatosis and hypertriglyceridemia (Baranowski, 2008).

2.2.1 Structure of LXRs

LXRs consist of four domains. First is the highly variable N-terminal region. It contains the ligand-independent activation function (AF)-1 domain (Wójcicka, Jamroz-Wiśniewska, Horoszewicz & Bełtowski, 2007). This domain can initiate gene transcription without ligand but the transcriptional activation is usually very weak. Protein-protein interaction also occurs at AF-1 domain (Zollner et al., 2010). Secondly is DBD with approximately 76 amino acids and comes together with two zinc fingers. DBD is highly conserved between species and it plays a critical role in receptor dimerisation.

Third domain will be the LBD. It is hydrophobic and is needed for ligand binding and receptor dimerisation. Between DBD and LBD, there is a hinge region which set the two domains apart. Lastly, there is a C-terminal ligand-dependent transcription activation domain which holds the AF-2 domain. AF-2 domain promotes transcription activity upon ligand binding. Figure 2.1 showed the structure of human LXR-α (hLXR-α).

2.2.2 Ligands of LXRs

The oxidised derivatives of cholesterol or endogenous oxysterols are the natural ligands of LXRs (Chen, Chen, Head, Mangelsdorf & Russell, 2007). There are three sources of oxysterols, which are endogenous production through enzymatic reactions, endogenous production through non-enzymatic reactive oxygen species (ROS)-dependent cholesterol oxidation and dietary intake (Wójcicka et al., 2007; Otaegui-Arrazola, Menéndez-Carreño, Ansorena

& Astiasarán, 2010). Since the metabolic fate of oxysterols is higher than cholesterol, therefore changes in oxysterols reflect the changes in cholesterol balance (Oosterveer et al., 2010). In other words, LXRs act as the cholesterol sensor in our body.

It is important to know that cholesterol itself is not the ligand for LXRs. Oxysterols are exemplified by 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol and 24(S), 25-epoxycholesterol. Among them, 24(S), 25-epoxycholesterol in liver is the most potent ligand (Oosterveer et al., 2010). This substance is formed during cholesterol biosynthesis. The 22(R)-hydroxycholesterol is intermediate substance in steroid hormone synthesis while 24(S)-hydroxycholesterol is mainly found in the brain (Zhang & Mangelsdorf, 2002). Besides oxysterols, LXR can also be activated by synthetic ligands or agonists like T0901317 and GW3965 (Hong & Tontonoz, 2008; Zhao & Dahlman-Wright, 2010) (Figure 2.2). These two agonists are commonly used in experimental studies.

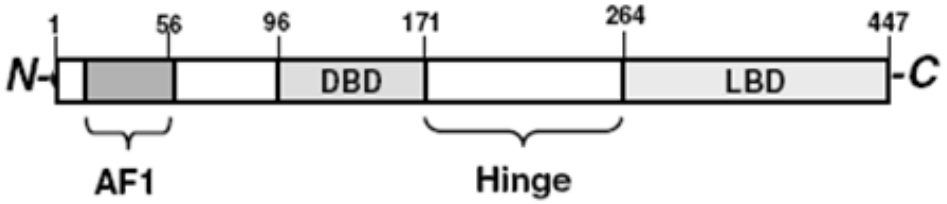


Figure 2.1 Schematic representation of hLXR-α

The hLXR-α consists of four domains, which are N-terminal region containing AF-1 domain, DBD that comes together with two zinc fingers, LBD and C-terminal region which holds the AF-2 domain. Between DBD and LBD, there is a hinge region which set the two domains apart (Modified from Chen, Bradley, Beavena & Tontonoza, 2006).

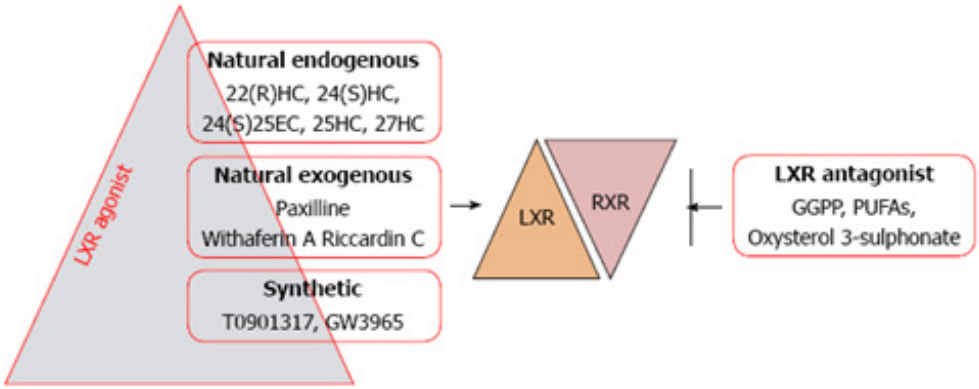


Figure 2.2 Classification of LXRs modulators

LXRs can be activated by natural endogeneous, natural exogeneous and synthetic ligands. On the other hand, action of RXR can be blocked by LXRs antagonists (Adapted from Dave & Kaul, 2010).

2.2.3 Activation of LXRs

LXRs regulate gene transcription through heterodimerisation with another NR, the Retinoid X Receptor (RXR), which is activated by 9-*cis* retinoid acid. The heterodimerisation of LXR and RXR is very specific and no other NR is reported to interact with LXR (Willy et al., 1995). Dimerisation enhanced the binding site affinity, specificity and diversity of NR (Germain et al., 2003). LXR/RXR is known as permissive heterodimer which means that it can be activated by ligand of either partner (Tontonoz & Mangelsdorf, 2003). Simultaneous binding of ligands of both receptors will generally produce a stronger response than ligand from one partner (Wójcicka et al., 2007).

Upon ligand binding, LXR and RXR undergo nuclear translocation. Subsequently, both the NRs formed obligate heterodimers and bind to the specific LXR response element (LXRE) found within the gene promoter (Mello, 2010). LXRE comprised of a variant of the idealised sequence AGGTCA(N_4)AGGTCA, which is a hexameric direct repeats separated by four nucleotides (DR-4) (Hong, Rho, Kim & Lee, 2010). In LXRs target genes, the consensus LXRE contains few invariant nucleotides in each half site and the nucleotides at other positions vary (Edwards, Kennedya & Mak, 2002).

Post-translational modification leads to structural modification of LXR/RXR complex which will modify the affinity for certain co-repressor (CR) or co-activator (CA). CR is a large protein that has high homology in repression domain at N-terminal region and in receptor interaction domain at the C-terminal region. Nuclear receptor co-repressor (N-CoR) and silent mediator of

retinoic acid receptor and thyroid receptor (SMRT) had been reported to have interaction with LXRs (Hu, Li, Wu, Xia & Lala, 2003). They suppressed transcription through histone deacetylase recruitment.

In contrast, CA is a protein that bound to the DBD of a transcriptional factor to enhance gene expression. They worked through the recruitment of histone acetyltransferase. Peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PPARGC1) (Oberkofler, Schraml, Krempler & Patsch, 2003) and transformation/ transcription domain-associated protein (TRRAP) (Unno et al., 2005) are some of the CAs of LXRs.

Generally, presence of oxysterol or 9-*cis* retinoid acid activates LXRs. This occurs through the release of histone deacetylase-containing CR complex and recruitment of histone acetylase-containing CA (Kim et al., 2009). These events leads to the activation of LXRs target genes to prevent cholesterol overload in cells. On the other hand, CRs remained in position when there is absence of ligand and LXR will bind to the target genes promoter to suppress the transcriptional activities (Hu et al., 2003).

Recently, LXRs has become the subject of many studies due to its potential anti-atherosclerotic and anti-inflammatory effects. Data showed promising prospect of LXRs as the target for drug development for a range of diseases such as cardiovascular diseases, hyperlipidemia and diabetes.

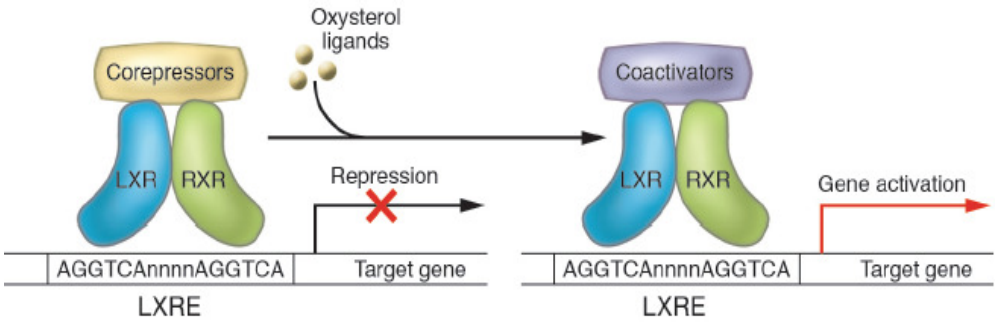


Figure 2.3 Transcriptional control by LXR

Upon ligand binding, LXR and RXR undergo nuclear translocation and formed obligate heterodimers. Subsequently, LXR/RXR complex bind to the specific LXRE on DNA, which is usually found within target gene promoter and activate gene transcription (Adapted from Zelcer & Tontonoz, 2006).

2.3 LXR-α

There are two isoforms of LXR, namely LXR-α (NR1H3) and LXR-β (NR1H2). Human LXR-α (hLXR-α) gene is found on chromosome 11p11.2 while human LXR-β gene is located on chromosome 19q13.3 (Zhao & Dahlman-Wright, 2010). DBD and LBD of both isoforms are 80% identical in their amino acid sequence (Zhao & Dahlman-Wright, 2010; Cronican et al., 2010).

In terms of distribution, LXR-α is highly expressed in liver and at lower level in other metabolically active tissues like small intestine and kidney (Fievet & Staels, 2009). As for LXR-β, it is ubiquitously found in our body with higher levels in developing brain. Reports have indicated that LXR-α is the main regulator of hepatic lipid metabolism whereas LXR-β controls energy and glucose metabolism (Zelcer & Tontonoz, 2006; Baranowski, 2008; Oosterveer et al., 2010). Another difference between these two isoforms is that LXR-α can be activated by thyroid hormone but not LXR-β (Hashimoto et al., 2007).

Researchers found that alternative splicing of hLXR-α resulted in another two isoforms of LXR-α, termed LXR-α2 and LXR-α3 (Chen, Beaven & Tontonoz, 2005; Wójcicka et al., 2007). These two new variants of hLXR-α have lower expression compared to LXR-α1 in most of the tissue. However, LXR-α2 is the main isoform found in testis (Dave & Kaul, 2010). Due to some structural differences, LXR-α2 has lower transcriptional activity than that of LXR-α1 whereas LXR-α3 is transcriptionally inactive as it is unable to bind ligand (Figure 2.4).

Furthermore, there are three LXRE in hLXR-α (Li et al., 2002; Hashimoto et al., 2007). Type I LXRE has strong affinity for both LXRs activity. For the other two LXREs (Type II LXREs), they are identical and are selectively bound to LXR-α. The Type II LXREs are found in *Alu* repeats and conserved within hLXR-α promoter (Figure 2.5).

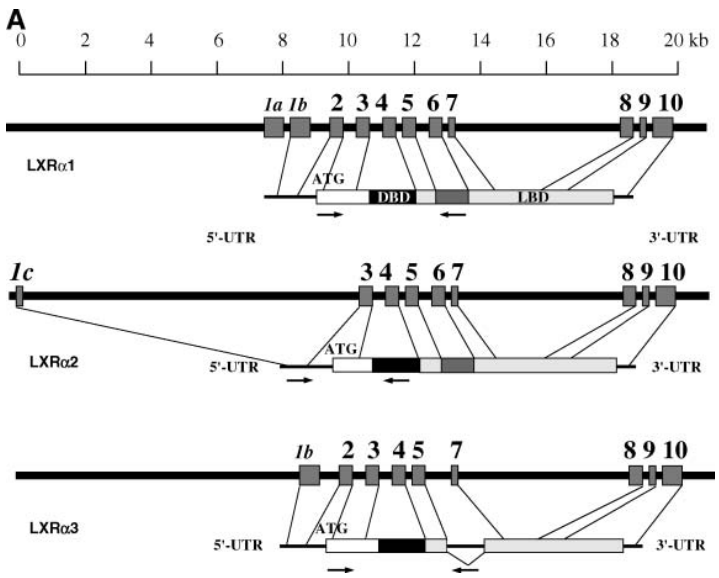
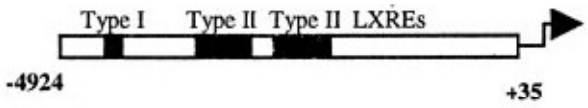


Figure 2.4 Schematic representation of genomic structure and protein structure of the three hLXR-α isoforms

(Adapted from Chen et al., 2005)



Type I LXRE	**-4139**	**TGAA TGACCA GCAG TAACCT CAGCA**
Type II LXRE	**-3290**	**CTCC TGACCT CAAG TGATCC ATCTG**
Type II LXRE	**-2918**	**CTCC TGACCT CAAG TGATCC ACCCG**

Figure 2.5 Schematic illustration of three LXREs within hLXR-α promoter

The actual sequence at position for Type I LXRE is -4139/-4115, while for Type II LXREs are -3290/-3266 and -2918/-2894 (Adapted from Li et al., 2002).

2.4 LXR-α and Cholesterol Metabolism

LXR-α is the master regulator of body cholesterol homeostasis (Song & Liao, 2001). Excess cholesterol activates LXRs and resulting in three outcomes, which are removal of cholesterol from cells through reverse cholesterol transport (RCT), inhibition of intestinal absorption and decreased cholesterol biosynthesis and uptake.

2.4.1 Reverse Cholesterol Transport (RCT)

RCT is a process whereby excess cholesterol in peripheral cells is transported by HDL back to liver for excretion (Rader, 2007). The first step of RCT involved the cholesterol uptake by specific acceptors like ATP-binding cassette (ABC) superfamily transporters. Next, the effluxed cholesterol undergoes esterification, catalysed by lecithin: cholesterolacyltransferase (LCAT) and shifts to the centre of the high density lipoprotein (HDL) particle. Subsequently, cholesterol and phospholipids transfer and acquisition of apolipoproteins (Apo) released from triglycerides (TG) lipolysis occurred. As such the nascent HDL is remodelled into mature HDL and their cholesterol is taken up by the liver.

Data revealed that a number of ABC superfamily transporters are direct targets of LXR-α. They are ABCA1, ABCG1, ABCG5 and ABCG8 (Miao et al., 2004). These transporters aid in cholesterol efflux, thereby reducing the low density lipoprotein (LDL) level and increasing the HDL level. ABCA1 and ABCG1 are highly expressed in liver cells, intestinal cells and macrophages. ABCA1 plays an important role in the efflux of cholesterol and phospholipids

to lipid-poor lipoprotein like ApoA-I (Oosterveer et al., 2010). On the other hand, the role of ABCG1 is not clearly known. It is suggested that ABCG1 may offer an additional pathway for cholesterol efflux or works together with ABCA1. As for ABCG5 and ABCG8, they are exclusively found in liver and gut. They enhance biliary cholesterol excretion (Tontonoz & Mangelsdorf, 2003).

Mutation in ABCA1 and ABCG5 or ABCG8 is implicated in Tangier disease and β-sitosterolemia, respectively (Schmitz & Langmann, 2005). Tangier disease is characterised by absence of plasma HDL and accumulation of cholesterol esters in reticuloendothelial cells (Zhu et al., 2005) whereas β-sitosterolemia causes increased body cholesterol and toxic plant sterols absorption and decreased biliary sterol secretion, eventually leads to premature atherosclerosis.

In order to lower the cholesterol load in cells, the cholesterol first needs to be redistributed from intracellular compartment to the plasma membrane. This is done by Niemann-Pick C1 (NPC1) and C2 (NPC2) proteins. LXRs agonists had been shown to up-regulate the expression of these two proteins (Rigamonti et al., 2005). Defect of either NPC1 or NPC2 resulted in Niemann-Pick C (NPC) disease, a fatal neurodegenerative disease characterised by the intracellular accumulation of cholesterol and other lipids (Hu, Zheng & Wang, 2010). Moreover, LXRs also positively modulate the expression of scavenger receptor type B1 (SR-B1). It is a specific HDL receptor mainly found in liver.

LXRs ligand was showed to induce the promoter activity and protein level of SR-B1 (Malerød, Juvet, Hanssen-Bauer, Eskild & Berg, 2002).

Meanwhile, LXRs also control the expression of extracellular cholesterol acceptors like ApoA-I and ApoE. ApoA-I is largely found in HDL. Observation showed that activation of LXRs significantly improved the expression of ApoA-I mRNA and protein (Lee, Tauscher, Seo, Oram, & Kuver, 2003). On the other hand, ApoE is a high-affinity ligand of the low density lipoprotein receptor (LDLR). It promotes the incorporation of cholesterol into lipid poor HDL via ABCA1. ApoE expression was showed to increase after T0901317 treatment in hippocampus and cerebral cortex in animal model (Liang et al., 2004). This condition also occurred in adipose tissues and macrophage but not in liver (Dave & Kaul, 2010).

In addition, LXRs influence the activity of lipoprotein remodelling enzymes or proteins (Zhang & Mangelsdorf, 2002). Finding showed that LXRs agonist induced lipoprotein lipase (LPL) expression in liver and macrophage. LPL catalyses the hydrolysis of TG-rich lipoproteins such as VLDL and chylomicrons. LXRs also regulate the expression of cholesterol ester transfer protein (CETP), which mediates the transfer of HDL cholesterol esters to Apo-B containing particles in exchange for TG (Oosterveer et al., 2010). LXRs activation in HepG2 cells leads to increase plasma level of phospholipids transfer protein (PLTP). PLTP is a protein that aids in the transport of phospholipids from TG-rich VLDLs and chylomicron remnants to HDLs. Reports indicated that LXRs are vital to PLTP expression and it was also

showed that a high-affinity LXRE is found within the PLTP promoters (Laffitte et al., 2003b).

2.4.2 Inhibition of Intestinal Absorption

In terms of intestinal cholesterol absorption, the key player is Niemann-Pick C1 Like 1 protein (NPC1L1) expressed in the brush border membranes of enterocytes (Fievet & Staels, 2009). It is an important sterol transporter in our body. Its malfunction can affect body cholesterol homeostasis. NPC1L1 shared 50% sequence homology with NPC1 and is negatively modulated by LXRs. NPC1L1 consists of 13 transmembrane domains and a sterol sensing domain (Davis et al., 2008). Study demonstrated that administration of LXRs agonist reduced NPC1L1 expression and hence reduced intestinal cholesterol absorption (Baranowski, 2008).

Besides their involvement in RCT, ABCG5 and ABCG8 also limit cholesterol absorption in intestine (Zelcer & Tontonoz, 2006). Study demonstrated that LXRs agonist treatment resulted in 3-fold increment in biliary cholesterol concentration and 4-fold increment in fecal sterol excretion in wild-type mice compared to ABCG5 and ABCG8 knockout mice (Yu et al., 2003).

2.4.3 Reducing Cholesterol Biosynthesis

Treatment with LXRs agonist in mice uncovers the role of LXRs in controlling the expression of lanosterol 14α-demethylase and squalene synthase (Wang et

al., 2008) in liver. This suggested that LXRs may inhibit cholesterol biosynthesis in the liver. Yet, cholesterol efflux followed LXRs activation may send a negative feedback signal to increase the cholesterol biosynthesis. Thus, more research is needed to confirm the relationship between LXRs and enzymes involved in cholesterol biosynthesis.

2.5 LXRs and Bile Acids Metabolism

Bile acids are produced from cholesterol in the liver. Its synthesis and secretion is the main route of cholesterol elimination. The rate-limiting enzyme involved in the conversion of cholesterol to bile acids is cholesterol 7α-hydroxylase (CYP7A1) (Chiang, 2004). Expression of CYP7A1 is mediated by LXRs, with LXR-α playing the dominant role (Peet et al., 1998). Nevertheless, human CYP7A1 is not a target of LXRs as *CYP7A1* gene does not contain LXRE (Chiang, Kimmel & Stroup, 2001; Handschin & Meyer, 2005).

Besides their involvement in RCT, ABCG5 and ABCG8 also take part in bile acids metabolism (Yu et al., 2003; Plosch et al., 2004). These two transporters aid in the hepatobiliary secretion of cholesterol and fecal sterol excretion. Recently, report showed that human uridine diphosphate glucuronosyl transferase 1A3 (*UGT1A3*) gene is up-regulated by LXR-α (Barbier, Trottier, Kaeding, Caron & Verreault, 2009). UGT1A3 is an active enzyme engaged in the glucuronide conjugation of bile acids. This process produced metabolites that can be excreted urinally. Therefore, it may imply that LXR-α activation facilitates cholesterol removal through urinary excretion of bile acids

glucuronides. Study revealed that actions of LXRs are preventive against bile acids toxicity and cholestasis (Uppal et al., 2007). Figure 2.6 shows a representative diagram of the roles played by LXRs in cholesterol transport and metabolism.

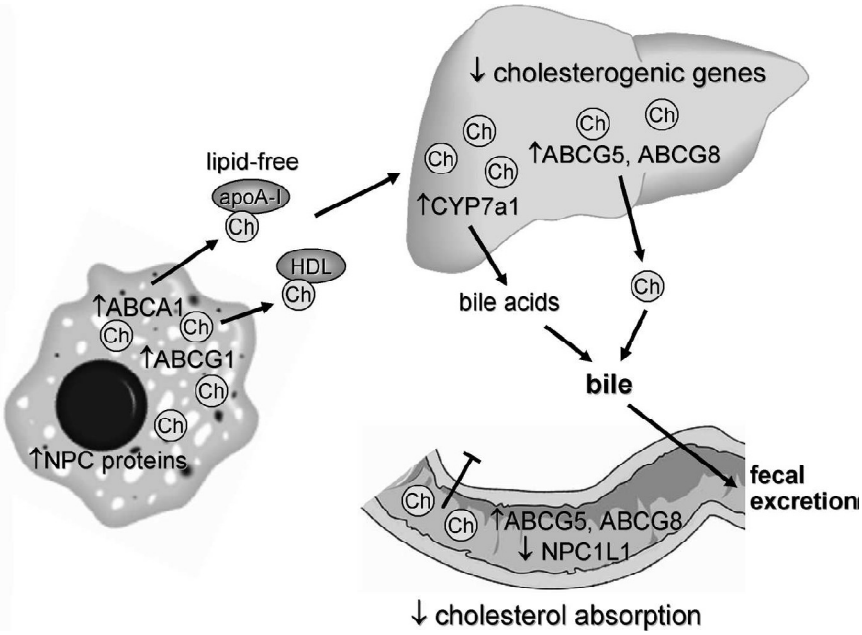


Figure 2.6 Roles of LXRs in cholesterol transport and metabolism

ABCA1, ABCG1, ABCG5, ABCG8, NPC proteins, NPC1L1 and CYP7A1 are LXRs' target genes (Adapted from Baranowski, 2008).

2.6 LXRs and Fatty Acids Metabolism

Despite positive effects, LXRs activation also results in lipogenesis. Hepatic and plasma TG level also increased markedly after LXRs agonist treatment. This effect is mediated by sterol regulatory element binding protein (SREBP-1c), which is a downstream mediator of LXRs action (Schultz et al., 2000). SREBP-1c is a transcription factor that controls the expression of enzymes involved in fatty acid synthesis.

Upon activation, SREBP-1c binds to the sterol response element (SRE) located in the promoter region of genes encoding lipogenic enzymes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and steroyl-CoA desaturase-1 (SCD-1). These enzymes are also regulated by LXRs (Joseph et al., 2002; Zhang, Yin & Hillgartner, 2001; Chu, Miyazaki, Man & Ntambi, 2006). Two LXREs are found within the SREBP-1c gene promoter (Yoshikawa et al., 2001) and deletion of SREBP-1c impaired fatty acids and TG synthesis. This proved that SREBP-1c is predominantly regulated by LXRs.

Looking at different aspect, LXRs-activated lipogenesis helps to convert the highly toxic free cholesterol to less toxic cholesterol esters and stored them in lipid droplets. This prevents lipotoxicity of free cholesterol (Schmitz & Langmann, 2005). In fact, the monounsaturated fatty acids produced by SCD are preferable substrates for acyl-CoA: cholesterol O-acyltransferase (ACAT), which mediates cholesterol esterification (Oosterveer et al., 2010).

2.7 LXRs and Carbohydrate Metabolism

Carbohydrate response element binding protein (ChREBP) is a glucose-sensitive transcription factor. When glucose level is high, it promotes the expression of lipogenic enzymes to convert the excess glucose to lipids. Cha and Repa (2007) found out that administration of aLXR agonists is capable to stimulate the expression of ChREBP in liver. This is due to the presence of two LXREs in promoter sequence of ChREBP gene.

On top of that, LXRs agonists are showed to improve insulin sensitivity in diabetic animal models (Cao et al., 2003). This resulted in decreased plasma glucose level. Finding uncovered that T0901317 and GW3965 reduced the expression of key enzymes involved in hepatic gluconeogenesis and glucose output. These enzymes included pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), fructose 1, 6-bisphosphatase and glucose 6-phosphatase (G6Pase) (Laffitte et al., 2003a).

Besides that, researchers found that glucose transporter (GLUT) 4 is a direct target of LXRs (Ulvena, Dalena, Gustafssonb & Nebb, 2005) and LXRE is found within the promoter of mice and human *Glut4* genes. Treatment with T0901317 is able to up-regulate the expression of GLUT4. Also, LXRs modulate insulin secretion as experiment showed that LXR agonist administration increased insulin level (Efanov, Sewing, Bokvist & Gromada, 2004). Under hyperglycaemic condition, LXR-α nuclear translocation is induced (Helleboid-Chapman et al., 2006).

2.8 LXRs and Innate Immunity

Recent studies revealed that LXRs possessed anti-inflammatory effects in many cell types (Joseph et al., 2004; Zelcer & Tontonoz, 2006). Activation of LXRs is able to down-regulate a series of pro-inflammatory genes such as cytokines, chemokines, adhesion molecules and acute phase proteins (Table 2.1).

LXRs agonists were shown to repress LPS-induced pro-inflammatory genes expression (Joseph et al., 2003; Zhang-Gandhi & Drew, 2007). Examples are interleukin-6 (IL-6), interleukin-1β (IL-1β) and inducible nitric oxide synthase (iNOS). The signalling process which causes the suppression of the pro-inflammatory genes is not clearly known as LXRE is not found in the promoter of repressed genes. It is proposed that this occurred through the inhibition of pro-inflammatory transcription factors like NF-κB (Zelcer & Tontonoz, 2006).

In addition, LXRs agonist treatment reduced cytokine-induced C-reactive protein (CRP) production in human hepatocytes. This is a due to the ability of LXRs to prevent the dissociation of the CR from CRP promoter region (Blaschke et al., 2006). Myeloperoxidase (*MPO*) gene is another gene reciprocally regulated by LXRs (Reynolds, Kumar & Piedrafita, 2006). High level of MPO is implicated in atherosclerosis development.

Table 2.1 Anti-inflammatory effects of LXRs agonists

Table 2.1 shows a list of genes that are suppressed as a result of LXRs activation (Modified from Jamroz-Wiśniewska, Wójcicka, Horoszewicz & Bełtowski, 2007).

Suppression of pro-inflammatory genes	
Cytokines	TNF-α IL-1β IL-6 G-CSF
Chemokines	MCP-1 MCP-3
Adhesion molecules	ICAM-1
Enzymes synthesising inflammatory mediators	COX-2 PGES-1 iNOS MPO
Matrix metalloproteinases and extracellular matrix proteins	MMP-9 OPN
Acute phase proteins	CRP SAP

Abbreviations: COX, cyclooxygenase; G-CSF, granulocyte colony-stimulating factor; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant proteins, MMP, matrix metalloproteinases; PGES, prostaglandin E synthase; SAP, serum amyloid P protein.

2.9 Tumour Necrosis Factor-alpha (TNF-α)

2.9.1 Structure, Biosynthesis and Receptors

TNF-α, or cachexin is a multifunctional cytokine with a wide range of roles, including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation (Tacer et al., 2007). It is produced by immune cells such as monocytes and macrophages during inflammation (Nan, Lin, Lumsden, Yao & Chen, 2005) and some non-hematopoietic cells (Shalita-Chesner, Katz, Shemer & Werner, 2001). It is the main regulator of inflammation and important player in cytokine network. The gene for TNF-α is encoded on chromosome 6p21.3.

TNF-α exists in two different forms. It is first synthesised as transmembrane TNF-α (tmTNF-α), which is a 26 kilodalton (kDa) protein composed of 212 amino acids (Kleinbongard, Heusch & Schulz, 2010). Normally, tmTNF-α comes in the form of homotrimers. Cleavage by TNF-α converting enzyme (TACE) resulting in a 17 kDa protein, which is known as the soluble TNF-α (sTNF-α). When three 17 kDa monomers bound together non-covalently, the biologically active form of TNF-α is produced. It is suggested that tmTNF-α involved in paracrine and autocrine signalling, whereas sTNF-α mediated the endocrine effects (Cawthorn & Sethi, 2008).

As high level of TNF-α can lead to lethal outcomes, thus the biosynthesis of TNF-α is highly regulated. In healthy individual, TNF-α is hardly detected (Tracey & Cerami, 1994). TNF-α brings out its function through two receptors. TNF-α receptor type 1 (TNFR1; CD120a) can be found on almost all cell types except erythrocytes. Second receptor is TNF-α receptor type 2 (TNFR2;

CD120b). It is mainly expressed on immune cells and heart. TNFR1 is activated by tmTNF-α and sTNF-α but TNFR2 can only be activated by tmTNF-α (Kleinbongard et al., 2010).

2.9.2 Biological Activities of TNF-α

TNF-α is a crucial communicating molecule in the body. Appropriate amount of TNF-α is needed to induce body host defence. When body is stimulated by harmful stimuli, TNF-α is induced through toll-like receptors (TLRs) and NF-κB signalling. This eventually leads to recruitment of immune cells to fight against the infectious agents. Study shown that administration of TNF-α is able to cause tumour necrosis in guinea pig model of sarcoma (Balkwill, 2009). Besides that, research done by Mattyasovszky et al. (2010) revealed that low level TNF-α has high proliferative effect on myofibroblast, which is involved in wound healing.

However, prolonged secretion of TNF-α is implicated in severe inflammation and even cancer. Research demonstrated that plasma TNF-α level was increased in some cancer patients. This is associated with the pro-tumour role of TNF-α. It is showed that cancer cells constitutively secrete tiny amount of TNF-α which can stimulate cancer cell growth and promote cancer metastasis. Moreover, TNF-α causes cachexia characterised by loss of protein, lipid and erythrocyte mass. This cachexia subsequently leads to insulin resistance and alteration in glucose metabolism (Tracey & Cerami, 1994). TNF-α can also

decrease the expression of thrombomodulin which plays a role in anti-coagulation and anti-inflammation (Nan et al., 2005).

In addition, TNF-α is involved in acute phase response (APR). During APR TNF-α alters lipid homeostasis by suppressing the genes involved in cholesterol excretion especially in RCT and enhanced the expression of cholesterogenic genes. Thus, this leads to reduced HDL-cholesterol and increased LDL-cholesterol (Tacer et al., 2007). Data also demonstrated that TNF-α decreases the expression of genes involved in fatty acids and bile acids metabolism, which contribute to changes in lipid profile (Kim et al., 2007b).

2.10 Kaempferol

2.10.1 Classification of Kaempferol under Flavonoids

Flavonoids are a group of polyphenols subclass which are ubiquitously found in vascular plant. There are six subclasses of flavonoids with kaempferol categorised under flavonol. Subtypes of flavonoids and their food source are shown in Table 2.2.

Kaempferol ($C_{15}H_{10}O_6$) (Figure 2.6) is a flavonoid compound commonly found in our diet. It makes up 30% of our dietary flavonoids intake (Niering et al., 2005). It is found in green tea, berries, and *Brassica* and *Allium* species. Robigenin, indigo yellow and campherol are some of its synonyms. The IUPAC name given to kaempferol is 3, 5, 7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one. Kaempferol got its name from one of its sources, aromatic

ginger (*Kaempferia galanga*). The molecular weight of kaempferol is 286.2363 g/mol. Figure 2.7 shows the basic structure of kaempferol.

Table 2.2 Subclasses of flavonoids and their food source

Flavonoids Subclass	Food Flavonoid	Food Source
Anthocyanidins	Cyanidin, delphinidin, pelargonidin	Red, purple and blue berries
Flavanols	Catechin, gallocatechin, epicathechin	Tea, red grapes, red wines
Flavanones	Naringenin, hesperetin, eriodictyol	Citrus foods
Flavones	Apigenin, luteolin	Green leafy spices
Flavonols	kaempferol, myricetin, quercetin, isorhamnetin	Nearly ubiquitous in foods
Isoflavones	Daidzein, genistein, glycitein, biochanin A	Soybeans, soy foods, legumes

(Adapted from Garcia-Lafuente, Guillamon, Villares, Rostagno & Martinez et al., 2009)

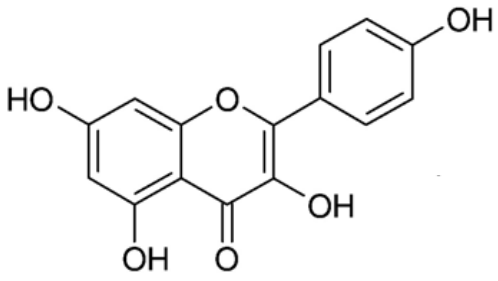


Figure 2.7 Structure of Kaempferol

(Adapted from Niering et al., 2005)

2.10.2 Biological Activities of Kaempferol

Studies showed that kaempferol has a wide range of beneficial health properties. Firstly it has anti-inflammatory properties. It suppresses the activation of NF-κB which will activate other pro-inflammatory mediators (Rios, Recio, Escandell & Andujar, 2009). Kaempferol also acts as an anti-oxidant by reducing the production of ROS (Leung et al., 2007). Besides that, Kaempferol plays a role in cardiovascular protection by suppressing the expression of adhesion molecules in human aortic endothelial cells (Garcia-Lafuente, Guillamon, Villares, Rostagno & Martinez, 2009). Specifically, kaempferol can inhibit TNF-α stimulated ICAM-1 and E-selectin expression in endothelial cells (Kumazawa, Kawaguchi & Takimoto, 2006). In addition, kaempferol is showed to have cancer chemoprotective property. As revealed in study done by Nguyen & et al. (2003), the pro-oxidant effect of kaempferol inhibited the growth and induced apoptosis in lung cancer cells.

2.11 Ascorbic Acid

Ascorbic acid, also known as Vitamin C, ascorbate, or L-ascorbic acid, is a water-soluble micronutrient identified in year 1932. The chemical formula of ascorbic acid is $C_6H_8O_6$, with molecular weight of 176.12 g/mol. Ascorbic acid is widely available in fresh fruits and vegetables (Jialal & Singh, 2006). Nevertheless, this vitamin can be lost during food processing as it is sensitive to oxygen, light, pressure and temperature (Oey, Verlinde, Hendrickx & Loey, 2006).

D-glucose or D-galactose is the precursor of ascorbic acid in majority of the plants and animals. Human is one of the species which cannot synthesise ascorbic acid from glucose due to mutation in the gene encoding L-gulonolactone oxidase, an essential enzyme in ascorbic acid biosynthesis (Naidu, 2003). Hence, we need to obtain this small molecule through dietary intake.

2.11.1 Metabolism of Ascorbic Acid

There are two forms of ascorbic acid. The reduced form is ascorbic acid whereas dehydroascorbic acid (DHAA) is the oxidised form. Ascorbic acid exerts its effects intracellularly and thus must be transported into the blood. Ascorbic acid is transported by sodium dependent vitamin C transporter (SVCT) 1 and SVCT2 (Padayatty & Levine, 2001; Belin, Kaya, Burtey & Fontes, 2010). SVCT1 is product of gene SLC23A1, encoded on chromosome 5q31.2-31.3. This transporter is expressed in intestine and kidney. As for SVCT2 which is found in plasma membranes of cells, is the product of gene SLC23A2, encoded on chromosome 20p12.2-12.3. Both SVCT1 and SVCT2 are very specific for ascorbic acid. DHAA is transported by GLUT1, GLUT3 and GLUT4. Their actions are inhibited by flavonoids (Rivas et al., 2008).

Generally, ascorbic acid is oxidised outside the cells. Once transported into the cell, the DHAA is quickly reduced to ascorbic acid. Active transport is the main transport mechanism for ascorbic acid. Though simple diffusion does occur in the mouth and stomach, but this only contributes to very small

percentage of total uptake. Ascorbic acid is not protein-bound and therefore undergoes filtration and reabsorption in kidney in healthy individuals. The ascorbic acid absorption rate is inversely correlated to the dosage administered (Padayatty & Levine, 2001). When cells are saturated with ascorbic acid, excess ascorbic acid will be excreted through urine. In adult human, the half life of ascorbic acid is approximately 10 to 20 days (Naidu, 2003).

2.11.2 Roles of Ascorbic Acid

Ascorbic acid is a well known antioxidant with low redox potential. It can act against a wide spectrum of free radicals like peroxide, hydroxyl, and superoxide radicals and peroxynitrile. Also, ascorbic acid regenerates other small molecules antioxidants like α-tocopherol and glutathione (GSH) from their radical species (Carr & Frei, 1999) and enhances mitochondrial function by improving the thiol status (Singh & Rana, 2010). Ascorbic acid is known as the most effective aqueous-phase antioxidant in human blood (Sadaba, Fernandez-Robredo, Rodriguez & Garcia-Layana, 2008). Hence, ascorbic acid may have protective roles against those age-associated diseases caused by free radicals exemplified by cancer (Hirakawa, Miura & Yagasaki, 2005; Drake et al., 1996; Jenab et al., 2006) and diabetes mellitus (Afkhami-Ardekani & Shojaoddiny-Ardekani, 2007; Hamden et al, 2009).

Ascorbic acid also functions as cofactor for hydroxylases and monooxygenase enzymes involved in collagen hydroxylation, carnitine and norepinephrine biosynthesis, tyrosine metabolism and peptide hormones amidation (Padayatty

& Levine, 2001; Abdul-Razzak, Alzoubi, Abdo & Hananeh, 2010). It accelerates the hydroxylation reaction of these enzymes and provides an optimal condition for enzymes functioning. Besides that, ascorbic acid is needed in the conversion of cholesterol to bile acids as it functions as the cofactor for the enzyme CYP7A1. Ascorbic acid deficiency decelerates this process causing cholesterol accumulation in liver.

Moreover, ascorbic acid is important in collagen maintenance. Without ascorbic acid, collagen hydroxylation is not complete and these improper fibers resulted in fragile blood vessels (Das, Ray, Snehlata, Das & Srivastava, 2006). It is also well-known that ascorbic acid improves the availability and absorption iron by enhancing the solubility of iron at alkaline pH and by maintaining the ferrous iron in its reduced state (Rana et al., 2010). High intake of ascorbic acid is always associated with treatment of illness especially the common cold. It is said so because it enhances the T-cell proliferation and thus stimulates our immune system, prevent T-cells apoptosis and maintain T-cell proliferation (Naidu, 2003).

Due to its anti-oxidative property, ascorbic acid is extensively used in pharmaceutical, cosmetic industries and preservative to prevent the undesired chemical changes as a consequence to oxygen exposure (Toyada-Ono et al., 2005). In food industries, ascorbic acid is always used as food additives, browning inhibitors, flavour stabilisers, dough modifiers and colour stabilisers (Naidu, 2003). Despite the beneficial roles, high intake of ascorbic acid is demonstrated to have pro-oxidant activity associated with production of anion

radical superoxide (O_2^-). Also, overdose of ascorbic acid may increase the risk of getting kidney stones and causes gastrointestinal disturbances (Abdul-Razzak et al., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Table 3.1 Materials used in the project and their suppliers

Materials	Suppliers
95% Ethanol	HmbG® Chemicals, Germany
Acetic acid, Bromophenol blue, Sodium acetate	Systerm, USA
Bromochloropropane (BCP), Ethidium bromide (EtBr), Formaldehyde, MOPS (sodium salt), Phosphate-buffered saline (PBS), Sodium acetate	Amresco, USA
Cryo-vial (1ml)	Nunc, USA
Cytokine: TNF-α	Chemicon International, USA
Ethylenediaminetetraacetic acid (EDTA), Kaempferol, Minimum essential medium (MEM), Sodium bicarbonate, Tissue culture grade DMSO, Trypsin-EDTA	Sigma Aldrich, USA
Foetal bovine serum (FBS)	i-DNA, Singapore
Forward primers, Reverse primers	Research Biolabs, Singapore
GeneRuler™ 100bp DNA ladder, 6X DNA Loading Dye (0.5 µg/µl)	Fermentas
Glycerol	QRëc™, USA
HepG2 cell line	American Type Culture Collection (ATCC), USA
Isopropanol	Merck, USA
L-Ascorbic-acid	Fisher Scientific, UK

Table 3.1 continued

Materials	Suppliers
L-Glutamine, Penicilin, Sodium pyruvate, Streptomycin	Gibco, USA
Quantifast SYRR Green RT-PCR Kit	QIAGEN, USA
RQ1 Buffer, RQ1 RNase-Free DNase, RQ1 DNase Stop Solution	Promega, USA
SaeKem® LE Agarose	Cambrex, USA
Sodium hydroxide (NaOH), pellets	R&M Chemical, UK
Tissue culture flask, Cell scrapers	Techno Plastic Products (TPP), Switzerland
Tris	Thermo Scientific, USA
TRI Reagent ®LS	Molecular Research Centre, Singapore

3.2 Cell Culture Media

3.2.1 Minimum Essential Medium (MEM)

MEM was prepared following the composition shown in Table 3.2. The medium was filter sterilised.

Table 3.2 Composition of MEM (per litre)

Solution	Composition
MEM powder, containing Earle's balanced salts (cell culture reagent) with 2.0 mM L-Glutamine	9.5 g
Sodium bicarbonate, 1 mM	2.2 g
Non-Essential amino acid, 0.1 mM	10 ml
Penicilin, 100 U/ml	10 ml
Sodium pyruvate, 1 mM	10 ml
Streptomycin, 100 µg/ml	10 ml
Sterilised deionised water	Top up to 1L

3.2.2 Phosphate-Buffered Saline (PBS)

Phosphate-buffered saline powder was dissolved in 1 litre of deionised water and was autoclaved at 121°C for 15 minutes at the pressure of 975 kPa.

3.3 Stock Solutions

Stock solutions used for RNA electrophoresis were prepared according to Table 3.3.

Table 3.3 Solutions for RNA Electrophoresis

Solution	Composition
RNA Loading Dye	50% (v/v) Glycerol, 1 mM EDTA, pH 8.0, 0.25% (w/v) Bromophenol blue
20X MOPS	400 mM MOPS (sodium salt), 100 mM Sodium acetate, 10 mM EDTA, pH 8.0, pH 7.0

3.4 Glassware and Plasticware Preparation

All glassware and plasticware (pipette tips, microcentrifuge tubes and centrifuge tubes) were autoclaves at 121°C for 15 minutes at the pressure of 975 kPa prior to use. The solutions used in handling of RNA were also autoclaved using the same conditions.

3.5 Preparation of Ascorbic Acid

Ascorbic acid stock solution with 20 mM concentration was prepared by adding 0.0704 g of L-ascorbic acid to 20 ml of sterile deionised water. The solution was stored in -20°C.

3.6 Cell Culture Techniques

3.6.1 Cell Culture Maintenance

HepG2 cells were grown in Eagle's MEM. The medium was supplemented with 2.0 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM of non-essential amino acid and 1.5 µg/ml

sodium bicarbonate, with 10% (v/v) heat-inactivated (30 minutes, 65°C), filtered sterilised FBS. The cells were maintained in a humid incubator of 5% (v/v) CO_2 at 37°C. The cell culture medium in the tissue culture flasks was replaced every two or three days.

3.6.2 Subculturing of Cells

Subculturing of cells was performed after the cells achieved 80% confluence. Initial medium in the tissue culture flasks was discarded and the cells were washed twice with PBS to remove all traces of serum which contained trypsin inhibitor. Then, 1 ml of 0.25 % (w/v) trypsin-EDTA was added to the cells and the culture flasks was incubated in 5% (v/v) CO_2 incubator at 37°C, for 10 to 15 minutes to allow the detachment of the cells from the growth surface. After incubation, the cells were observed under inverted phase contrast microscope to ensure all cells were fully detached. Next, 2 ml of complete growth medium was added to achieve a final volume of 3 ml. The medium was then transferred into a sterile 15 ml centrifuge tube and centrifuged at 500 *g* for 5 minutes. Following centrifugation, the supernatant was discarded and the pellet was resuspended in 2 ml of MEM if the cells were to be subcultured into two new flasks. One ml of the medium containing cells was plated onto each new tissue culture flasks with an addition of 2.6 ml of MEM and 0.4 ml of FBS. The cells were then incubated in incubator of 5% (v/v) CO_2 at 37°C.

CHAPTER 4

RESULTS

4.1 Culture of HepG2 cells

HepG2 cell line was derived from liver tissue of a 15 years old male Caucasian adolescent with hepatocellular carcinoma (American Type Culture Collection, 2010). HepG2 cells are of epithelial morphology and frequently used for metabolic studies of lipoprotein, cholesterol and bile acids (Javitt, 1990). The cells were cultured as mentioned in Section 3.6.1. Figure 4.1 showed the morphology of HepG2 cells.

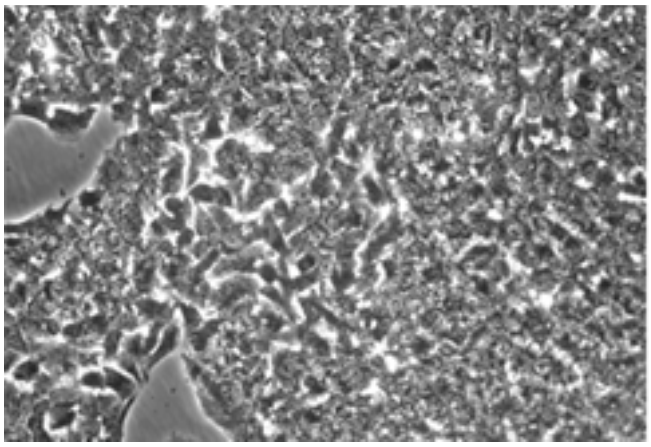


Figure 4.1 HepG2 cells cultured in 25 cm^2 tissue culture flask

HepG2 is a perpetual adherent cell line which was derived from the human liver tissue with hepatocellular carcinoma. These cells are epithelial in morphology (Adapted from American Type Culture Collection, 2009).

4.2 Isolation of Total Cellular RNA

Isolation of total cellular RNA of HepG2 cells was performed using TRI Reagent ®LS (Section 3.7.1). A small amount of isolated RNA was subjected to 1% (w/v) denaturing agarose-formaldehyde gel electrophoresis (Section 3.7.3) to assess the integrity of total RNA. Figure 4.2 shows the agarose-formaldehyde gel image for the isolated RNA. The gel image shows clear bands of 28S rRNA and 18S rRNA with the intensity ratio of approximately 2:1. This indicates that the RNA is completely intact and was not degraded. The concentration and absorbance at 260 nm and 280 nm of the RNA was determined by using NanoDrop ND®-1000 spectrophotometer. The ratio of A_{260} /A_{280} was used to indicate the purity of RNA (Section 3.7.2). Table 4.1 and Table 4.2 showed the A_{260} /A_{280} value of the extracted RNA. As all the value was approximately 2, this indicateed that the isolated RNA was of pure quality.

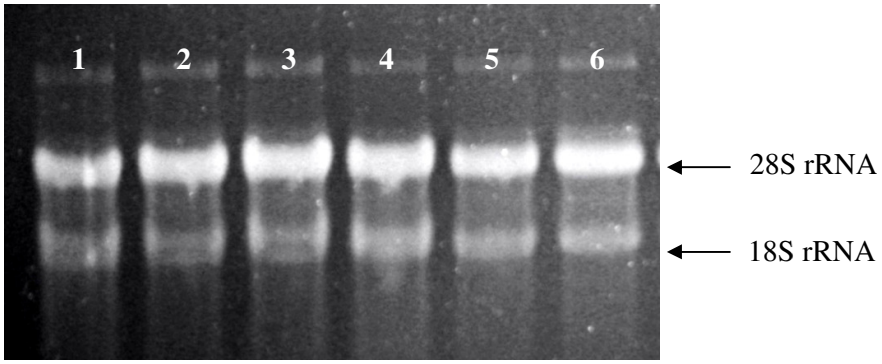


Figure 4.2 Denaturing agarose-formaldehyde gel electrophoresis of total RNA

A small volume of isolated RNA was subjected to 1% (w/v) denaturing agarose-formaldehyde gel electrophoresis. Lane 1, RNA isolated from untreated HepG2 cells; Lane 2 to 5, RNA isolated from HepG2 cells treated with 20 ng/ml of TNF-α and increasing dose of ascorbic acid (0, 15, 150 and 1500 μM); Lane 6, RNA isolated from HepG2 cells treated with 1500 μM ascorbic acid. The observation of 28S rRNA and 18S rRNA bands with an intensity ratio of 2:1 showed that the isolated RNA was intact and free from degradation.

Table 4.1 The concentration and A_{260} /A_{280} ratio of isolated RNA from cells treated with 20 ng/ml TNF-α and different concentration of kaempferol

The A_{260}, A_{280}, ratio of A_{260} /A_{280} and concentration of isolated total cellular RNA were determined by using NanoDrop ND®-1000 Spectrophotometer. The value of A_{260} /A_{280} which fell in 1.8 to 2.0 indicating that the RNA was intact and pure.

RNA Sample	A_{260}	A_{280}	A_{260} /A_{280}	Concentration (ng/µl)
1. Untreated	22.419	10.830	2.07	696.75
2. 20 ng/ml TNF-α	29.645	13.857	2.07	1145.81
3. 20 ng/ml TNF-α + 1.0 µM kaempferol	28.521	13.241	2.08	1140.83
4. 20 ng/ml TNF-α + 5.0 µM kaempferol	20.149	9.626	2.09	805.95
5. 20 ng/ml TNF-α + 10.0 µM kaempferol	16.603	9.020	2.06	744.13
6. 20 ng/ml TNF-α + 20.0 µM kaempferol	22.438	10.729	2.09	897.52
7. 20.0 µM kaempferol	0.806	0.383	2.104	322.00

Table 4.2 The concentration and A_{260} /A_{280} ratio of isolated RNA from cells treated with 20 ng/ml TNF-α and different concentration of ascorbic acid

The A_{260}, A_{280}, ratio of A_{260} /A_{280} and concentration of isolated total cellular RNA were determined by using IMPLEN Nanophotometer. The value of A_{260} /A_{280} which fall in 1.8 to 2.0 indicating that the extracted RNA was intact and pure.

RNA Sample	A_{260}	A_{280}	A_{260} /A_{280}	Concentration (ng/µl)
1. Untreated	2.122	1.204	1.771	844
2. 20 ng/ml TNF-α	1.963	1.083	1.813	785
3. 20 ng/ml TNF-α + 15 µM ascorbic acid	2.078	1.170	1.781	828
4. 20 ng/ml TNF-α + 150 µM ascorbic acid	1.616	0.850	1.909	644
5. 20 ng/ml TNF-α + 1500 µM ascorbic acid	2.352	1.279	1.832	945
6. 1500 µM ascorbic acid	1.927	1.203	1.604	769

4.3 Real Time RT-PCR

The main aim of this study is to analyse and quantify the effect of kaempferol and ascorbic acid on LXR-α mRNA expression HepG2 cells. As such, Real Time RT-PCR was performed on iCycler iQ5 Real Time PCR Detection System (Bio-Rad) using Quantifast SYBR Green RT-PCR (QIAGEN), according to manufacturer's instructions (Section 3.8). The protocol and primers used were stated in Section 3.8 and shown in Table 3.4, respectively. In order to get more accurate results, triplicates were used for each sample for both reference and target gene. β-actin was chosen as the housekeeping gene to determine the relative mRNA expression of the target gene.

The graphical representation of logarithmic scale in LXR-α in Real Time RT-PCR amplification was shown in Figure 4.3 and Figure 4.4. The graphs show cycle number along the X-axis while Y-axis shows the arbitrary fluorescent units which is the fold increase over background fluorescence. A threshold level is set sufficiently above background and the number of cycles required to reach threshold which is known as C_T value, is determined.

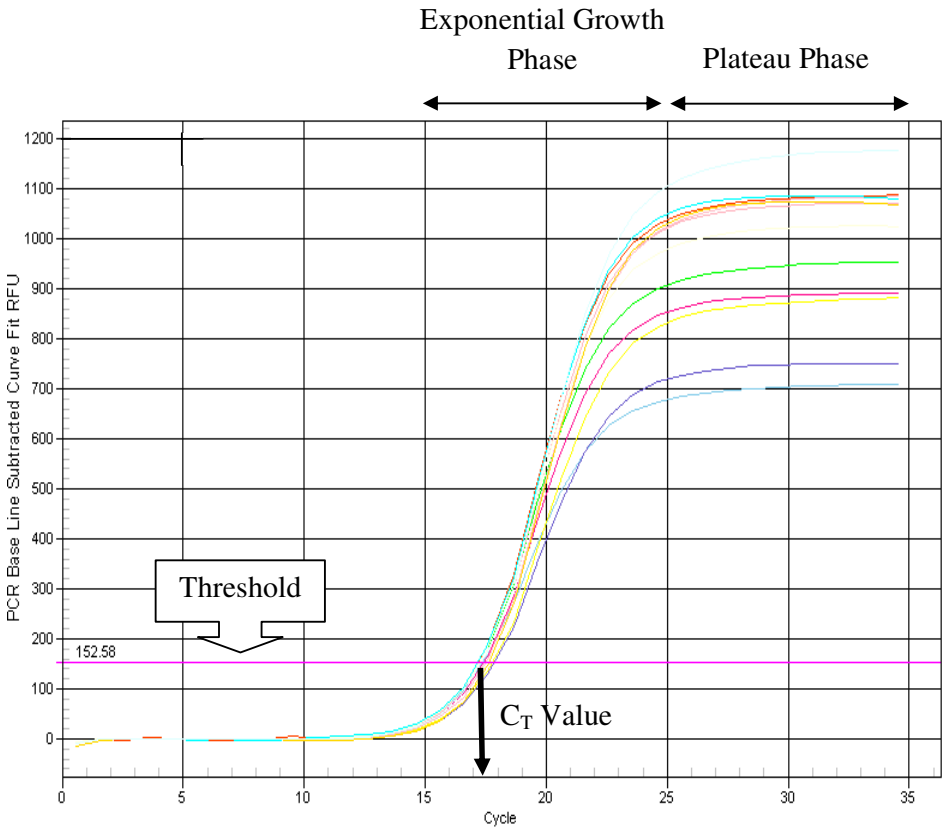


Figure 4.3 Graphical representation of logarithmic scale in Real Time RT-PCR amplification of reference gene, β-actin

PCR amplification graph was produced as a sigmoid curve in which cycle number was shown along the X-axis and arbitrary fluorescence units (which represented the fold increase over background fluorescence) are shown on the Y-axis. A threshold level was set sufficiently above background and the number of cycles required to reach threshold, C_T values, are registered.

PCR Amp/ Cycle Chart

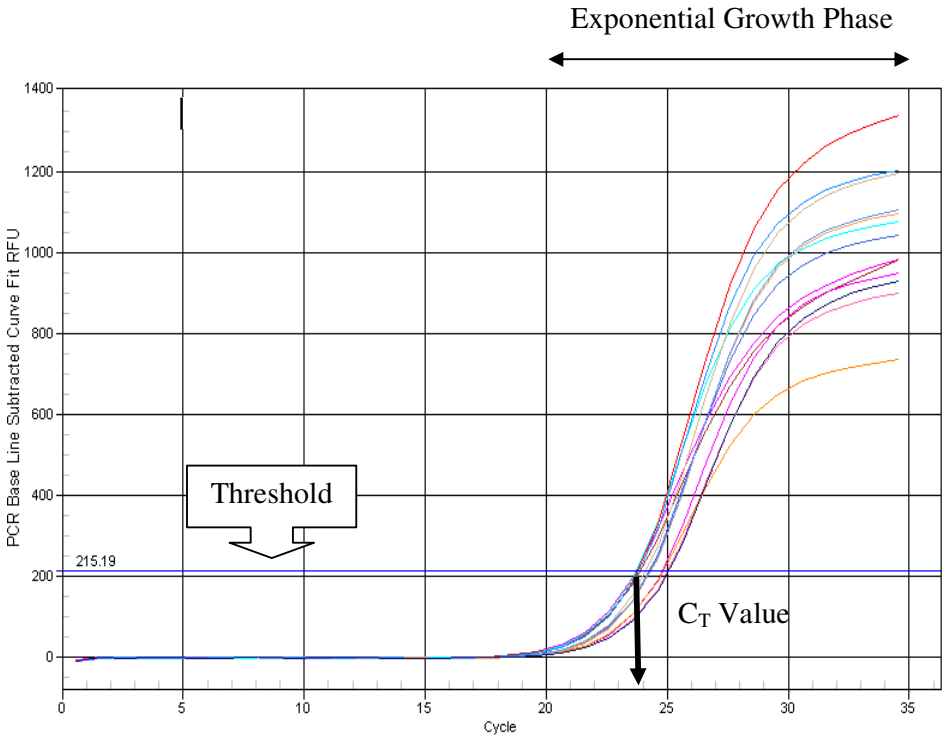


Figure 4.4 Graphical representation of logarithmic scale in Real Time RT-PCR amplification of target gene, LXR-α

PCR amplification graph was produced as a sigmoid curve in which cycle number was shown along the X-axis and arbitrary fluorescence units (which represented the fold increase over background fluorescence) are shown on the Y-axis. A threshold level was set sufficiently above background and the number of cycles required to reach threshold, C_T values, are registered.

A primer specificity test was performed to calculate the target specificity of primer through analysis of its melting curve chart and gene expression data prior to analysis of dose-response of LXR-α expression under stimulation of kaempferol and ascorbic acid in TNF-α stimulated HepG2 cells. Figure 4.5 shows the graphical representation of a melting curve analysis in Real Time RT-PCR during primer specificity test. A melting curve was obtained after PCR amplification whereby the temperature was raised by a fraction of a degree and the change in fluorescent was measured. The double-stranded DNA will separate at the melting point and the fluorescence decreases rapidly. The software plots the rate of change of the relative fluorescent units (RFU) with time (T) (-d (RFU)/ dT) on the Y-axis versus the temperature on the X-axis. The peak shows the melting temperature (T_m) of the PCR product. The melting temperature of β-actin and LXR-α was determined as shown in Figure 4.5 and Figure 4.6, respectively. The peak that was obtained indicated that the Real Time RT-PCR was specific with successful amplification of one PCR product.

Melt Curve Peak Chart (-dF/ dt vs T)

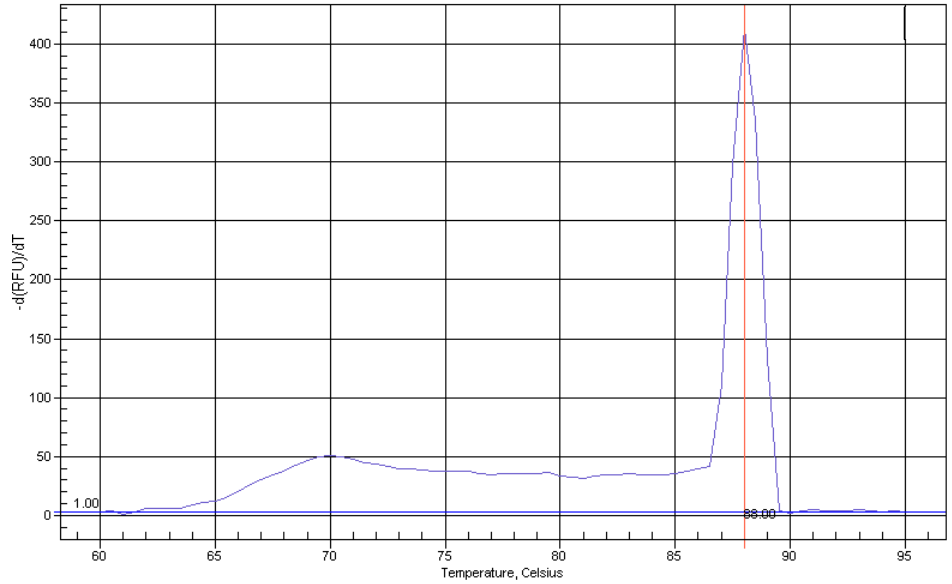


Figure 4.5 Graphical representation of a melting curve analysis to determine the primer specificity of β-actin during Real-Time RT-PCR

After PCR amplification, the Real-Time system was programmed to perform a melt curve, in which the temperature was raised by a fraction of a degree and the change in fluorescent was measured. The double-stranded DNA will separate at the melting point and the fluorescence decreases rapidly. The software plots the rate of change of the relative fluorescent units (RFU) with time (T) (-d (RFU)/ dT) on the Y-axis versus the temperature on the X-axis. The peak at the melting temperature (T_m) was generated for β-actin, which was around 88°C.

Melt Curve Peak Chart (-dF/ dt vs T)

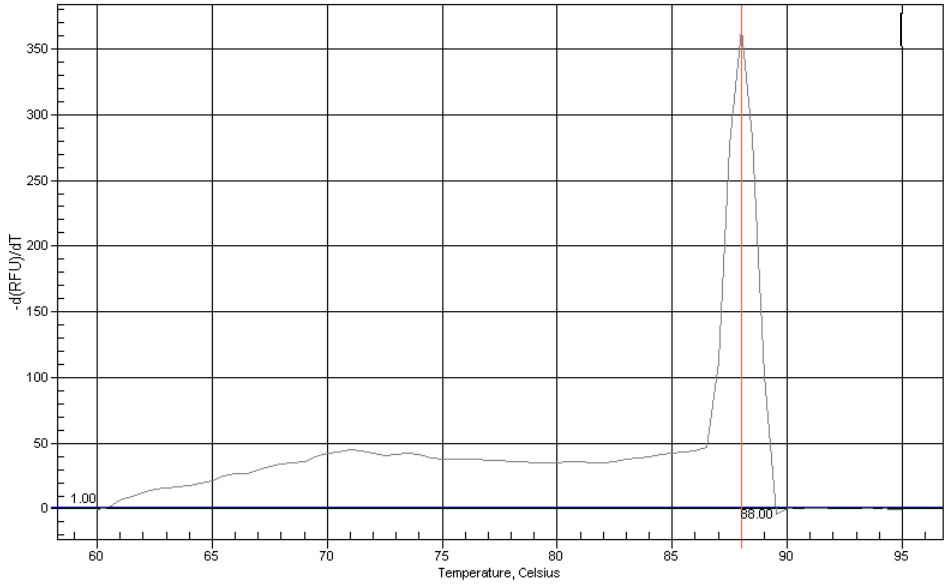


Figure 4.6 Graphical representation of a melting curve analysis to determine the primer specificity of LXR-α during Real Time RT-PCR

After PCR amplification, the Real-Time system was programmed to perform a melt curve, in which the temperature was raised by a fraction of a degree and the change in fluorescent was measured. The double-stranded DNA will separate at the melting point and the fluorescence decreases rapidly. The software plots the rate of change of the relative fluorescent units (RFU) with time (T) (-d (RFU)/ dT) on the Y-axis versus the temperature on the X-axis. The peak at the melting temperature (T_m) was generated for LXR-α, which was around 88°C.

CHAPTER 5

DISCUSSION

5.1 Review

There were few objectives in this study. First, is to investigate and quantify the effect of kaempferol on LXR-α mRNA expression in TNF-α stimulated HepG2 cell line. Second, is to investigate and quantify the effect of ascorbic acid on LXR-α mRNA levels in HepG2 cells. Lastly, this project aimed to compare and analyse the roles of kaempferol and ascorbic acid in hepatic LXR-α regulation.

5.2 HepG2 Cells as Study Model

HepG2 is a cell line that is free from known hepatotropic viral agents and is frequently used as a model for liver function study. Since its introduction in year 1979, this cell line had been applied in a wide range of studies involving liver function as it closely resembles the behaviour of human hepatocytes in culture (Alía et al., 2006). It retains many functions of normal liver cells such as synthesis of albumin, lipoproteins and other liver-specific proteins (Mooradian, Haas & Wadud, 2006). HepG2 cell line is an ideal model for research study as it has infinite life-span, stable phenotype and is easy to handle.

Moreover, HepG2 cells can respond to cytokines treatment in similar manner to liver cells (Jura, Wegrzyn, Zarebski, Wladyka & Koj, 2004) and is capable to express various cytokine genes such as TNF-α, interferon γ (IFN-γ) and macrophage colony-stimulating factor (M-CSF) (Stonans, Stonane & Wiederhold, 1999). Also, HepG2 cells can express a wide array of liver-specific metabolites especially those involve in cholesterol and TG metabolism (Javitt, 1990). Since liver is the organ where LXR-α is highly expressed (Fievet & Staels, 2009), and LXR-α is known to be involved mainly in cholesterol metabolism, HepG2 cell line is thus chosen for this study.

5.3 Isolation of Total Cellular RNA

RNA is an unbranched polymer which consists of purine and pyrimidines, connected by phosphodiester bonds. RNA is hard to be isolated as it is easily degraded either mechanically or chemically. The presence of the RNase ubiquitously contributes to the difficulty in RNA isolation since it is not easy to be inactivated. Total RNA was isolated using TRI Reagent ®LS, which is a single-step method allowing simultaneous isolation of DNA, RNA and protein. This is an effective way to extract RNA and the yield is of high quality with little or no contaminants (Chomczynski & Sacchi, 1987).

TRI Reagent ®LS is a mixture of guanidine thiocyanate and phenol in a homogeneous solution (Rapley, 1998). Guanidine thiocyanate is a strong chaotrope, which is a substance capable to disrupt the three dimensional structure of macromolecules. It can dissolve most of the cell components as to

release the RNA (Rapley, 1998). Both guanidine thiocyanate and phenol are strong denaturants which can inactivate RNase during the isolation process, thus maintaining the RNA integrity. Once extracted, RNA becomes relatively unstable (Bustin, Benes, Nolan & Pfaffl, 2005). Due to its short half-life and susceptibility to degradation by post-mortem processes, improper handling and storage, hence RNA extraction must be done cautiously and isolated RNA should be stored in -80°C.

5.4 RNA Integrity and Purity

Denaturing formaldehyde-agarose gel electrophoresis was carried out to determine the integrity of isolated RNA. RNA tends to form secondary and tertiary structures and this hinder its separation during electrophoresis (Farrell, 2010). Therefore, RNA was denatured prior to and during electrophoresis. This is accomplished by heating at 65°C before electrophoresis and addition of formaldehyde in the gel. Here, formaldehyde acts as RNA denaturants as it contains active hydrogen which aids in disrupting the secondary and tertiary structures of RNA.

Figure 4.2 showed two separate bands of RNA. Those are ribosomal RNA (rRNA) as they formed 80% of total cellular RNA. The upper band represents 28S rRNA while the lower band represents the 18S rRNA. The 28S and 18S rRNA showed fluorescence ratio of 2:1, which indicated that the extracted RNA was intact and free from degradation.

The purity of isolated RNA was measured spectrophotometrically. RNA was measured for absorbance at 260 nm (A_{260}) and 280 nm (A_{280}). RNA with ratio A_{260}/ A_{280} ~2.0 ± 0.1 is considered pure (Farrell, 2010). Ratio value below 2.0 suggested protein contamination which absorb at 280 nm. Table 4.2 showed absorbance reading below 2.0, this may be due to protein contamination during the isolation process. This can be eliminated through an additional step, where RNA samples are dissolved in nuclease-free buffer and extracted again with phenol: chloroform: isoamyl alcohol (25:24:1). Extraction is repeated again with chloroform. Besides, it is known that the pH of the diluents does affect the ratio value. Thus, to get a more accurate reading, it is encourage to dilute the RNA samples with Tris-EDTA or phosphate buffer so that the pH of RNA is maintained within 7.5 to 8.5 (Farrell, 2010).

5.5 Real Time RT-PCR

5.5.1 Basic Principles

Real Time RT-PCR has become a popular tool to quantify mRNA expression. It offers simultaneous gene expression measurement in different samples for a few genes and is useful when the number of cells is limited (Vandesompele et al., 2002). The procedure is easy to handle and has high throughput (Bustin, 2002). A major advantage over the conventional PCR is that Real Time RT-PCR can detect the amplification for each cycle along the reaction and give quantitative result. In contrast, conventional PCR measures the end-point product through agarose gel electrophoresis, which is tedious and time-consuming. Besides, Real Time RT-PCR has higher sensitivity and resolution

than the conventional PCR. In conventional PCR, products with similar or same size cannot be distinguished through the agarose gel electrophoresis. For Real Time RT-PCR, it can detect as little as two-fold change.

In Real Time RT-PCR, mRNA was reverse-transcribed into cDNA. Subsequently, the cDNA are amplified and quantification is done (McPherson & Møller, 2006). In this study, SYBR Green dye was used for Real Time PCR products detection. It is non-specific fluorescent intercalating agent which binds to the minor groove of DNA (Pfaffl, 2001). In solution SYBR Green exhibits little fluorescence. However, as it binds to the double-stranded DNA (dsDNA), its fluorescence increased significantly. The fluorescent signals from SYBR Green correspond to the number of amplified products.

SYBR Green is the cost-effective, sensitive and easy to use. It can bind to any dsDNA and thus design for a specific probe for target gene is not needed. Nevertheless, this property may also lead to over-estimation of product concentration as it can bind to non-specific products generated during the reaction. To overcome this limitation, specific primers for LXR-α were used for this study.

There are two quantification methods in Real Time RT-PCR. First is relative quantification where the relative expression of target gene to a reference gene is measured. The target gene mRNA levels can be normalised to total RNA, genomic DNA, reference gene rRNA, reference gene mRNA or alien molecules (Huggett, Dheda, Bustin & Zumla, 2005). Second is absolute

quantification which is based on an internal or external calibration curve. To obtain an accurate and reliable quantification, normalisation is required (Pfaffl, 2001).

Normalisation aims to eliminate the sampling difference in order to measure the real gene expression. Housekeeping genes can be found in all nucleated cells as they are necessary for cell survival (Pfaffl, 2001). Their expression in the tested cells should be stable throughout the research study or after experimental treatment (Vandesompele et al., 2002). Some examples of frequently used reference genes are β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S rRNA.

In this study, target gene expression is measured using relative quantification and β-actin was chosen as the housekeeping gene. Relative quantification is simple to handle and every stage of the Real Time RT-PCR can be controlled (Huggett et al., 2005) while β-actin it is expressed constantly throughout the treatment of TNF-α in HepG2 cell line (Chew et al., 2007). GAPDH was not selected as evidences showed that its mRNA level is highly variable even in cellular subpopulations of same pathological origin. Study showed that GAPDH should not be used as an internal control in most experiment (Bustin, 2002). However, study carried out by Vandesompele et al. (2002) demonstrated that there is no ideal and universal housekeeping gene. Thus, researchers are urged to search for suitable control gene for the experimental

system that one works on. Alternatively, multiple internal control genes can be used (Bustin et al., 2005).

5.5.2 PCR Amplification/ Cycle Chart

The PCR amplification chart for β-actin and LXR-α are shown in Figure 4.3 and 4.4, accordingly. At the early phase of reaction, no products are detected as the fluorescence signal is very low and did not reach the detection limit of the instrument. As the reaction continued, the fluorescent signals increase significantly, during the exponential phase. This is when the efficiency of PCR is close to 100%. Quantity of PCR products generated doubled in each cycle. When reaction reached plateau phase, the net synthesis is nearly zero due to depletion of components needed in the reaction such as primers and SYBR Green dye.

Cycle threshold (C_T) value is identified during the reaction. It indicates the cycle number where the reaction's fluorescence crossed the threshold. The C_T value is proportional to the number of template copies in the sample (Logan, Edwards & Saunders, 2009). The higher the amount of target at the beginning of the reaction, the fewer the amplification cycle are needed for the fluorescence signal to reach the threshold level and hence the lower the C_T value.

5.5.3 Melt Curve Analysis

Melt curve analysis was carried out after PCR amplification to test the product specificity of the reaction. Also, it will tell the presence of primer-dimers, splice variants and non-specific PCR products, if there is any (Logan et al., 2009). The melting temperature (T_m) of dsDNA is registered when 50% of the DNA became single-stranded. At this point, there will be a sudden decrease in fluorescent signals which indicates that the SYBR Green is released upon dsDNA dissociation. The T_m is dependent on the DNA length and guanidine and cytosine (GC) content.

A specific PCR product should have T_m above 80°C. As in this study, the T_m for both β-actin and LXR-α is the same, which is 88°C. Both graphs shown in Figure 4.5 and 4.6 demonstrated sharp peaks and this signified that the primers used are specific in amplifying the DNA products for β-actin and LXR-α.

5.7 Future Study

For future study, investigation on the actual molecular mechanisms that are involved in regulation of LXR-α mRNA expression by kaempferol and ascorbic acid is suggested. This allows a better understanding on how these two antioxidants and anti-inflammatory agents influence LXR-α gene expression.

Besides that, LXR-α expression in response to kaempferol and ascorbic acid treatment can be studied at the protein level to check if the mRNA expression

corresponds to the protein level. This could be used to validate whether there is any post-translational modification to LXR-α before the mRNA is translated into protein.

Also, more experiments investigating the effects of ascorbic acid or kaempferol can be done on other LXR-α isoforms. As discussed before, there are three isoforms of LXR-α as a result of alternative splicing. By revealing the expression of each isoforms, a more precise picture of LXR-α expression can be obtained.

CHAPTER 6

CONCLUSION

In this project, the main objectives were to investigate and quantify the effect of kaempferol and ascorbic acid on LXR-α mRNA expression in HepG2 cells treated with and without TNF-α using Real Time RT-PCR.

For the first part of the study where the effect of kaempferol was tested, results showed that LXR-α expression was suppressed to 0.648-fold after TNF-α treatment. Kaempferol treatment (1, 5, 10 and 20 μM) after TNF-α stimulation showed a gradual increase in LXR-α expression. At the highest concentration, LXR-α mRNA was expressed at 0.927-fold compared to control. Conversely, 20 μM of kaempferol alone did not bring any significant changes in the target gene expression, which is 1.048-fold.

In the second part of the project, ascorbic acid (15, 150 and 1500 μM) given after TNF-α treatment did not relieve the inflammatory effect of TNF-α, but synergistically suppressed the expression of LXR-α mRNA to 0.767-, 0.694- and 0.571-fold in a dose-dependent manner. In addition, treatment with 1500 μM alone suppressed LXR-α mRNA significantly to 0.320-fold compared to control sample.

In a nutshell, this study has successfully profiled that kaempferol, but not ascorbic acid was able to alleviate the TNF-α down-regulatory effects on LXR-α mRNA in HepG2 cells.

CHAPTER 7

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CHAPTER 8

APPENDICES

Appendix A

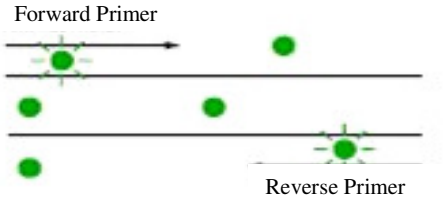
SYBR green dye assay

SYBR® GREEN I DYE ASSAY CHEMISTRY

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

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

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



4. **Polymerization completed**: When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.