STUDY ON THE TNF-α-STIMULATED NF-*κ*B PATHWAY REGULATING PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARα) EXPRESSION IN HEPG2 CELLS

By

LIM WYI SIAN

ABSTRACT

STUDY ON THE TNF-α-STIMULATED NF-κB PATHWAY REGULATING PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARα) EXPRESSION IN HEPG2 CELLS

LIM WYI SIAN

Peroxisome proliferator activated receptor-alpha (PPARα) is a ligand-activated transcription factor that belongs to the nuclear receptors family and it is involved in the regulation of inflammation and lipid and glucose homeostasis. Although TNF-α has been demonstrated previously to inhibit PPARα activity, the mechanisms underlying its regulation on PPARα in human liver cells are not completely understood. This study was aimed to systematically investigate the effects of TNF-α and its regulation on PPARα expression in HepG2 cells using Real-Time RT-PCR and western blot analysis. Here, TNF-α suppressed PPARα mRNA expression in a dose- and time-dependent manner at the level of gene transcription. A significant PPARα reduction to 0.67-fold was observed at 4 h in 20 ng/ml of TNF-α treatment. Prior to TNF-α treatment, pre-treatment of cells with 10 μM of Wedelolactone, an inhibitor of IKKα and IKKβ, for 2 h not only restored PPARα expression to more than its basal levels (1.68-fold), it also affected the expression of PPARα target genes ApoAI and ApoB. This indicates that NF-κB may act as a regulator that inhibits the induction of PPARα as a lipid metabolising gene. Furthermore, induction of PPARα expression in cells pre-treated with SC-514

and NF-κB activation inhibitor IV confirmed that the NF-κB pathway plays an important role in the suppression of PPARα expression in TNF-α-treated cells. TNF-α was shown to augment the activity of canonical NF-κB signalling pathway with an increase in active p50 protein levels, but not the proteolytic processing of the p100 subunit into p52 in the nucleus following stimulation with TNF-α by 15 min. Furthermore, TNF-α-induced phosphorylated IκBα (Ser32), IκBβ (Thr19/Ser23), and p105 (Ser933) were markedly reduced in Wedelolactone-treated cells. Although TNF-α-induced phosphorylation of p65 at Ser276, Ser468, and Ser536 had no effect on the translocation of the protein to the nuclear fraction, protein levels of p65 at Ser276 and Ser468 were shown to be more abundant when compared with the protein level of Ser536 in the nuclear fraction. Surprisingly, phosphorylation of p65 at Ser468 and Ser536 was severely abrogated with Wedelolactone inhibition, suggesting that Ser468 and Ser536, but not Ser276, may be mediating the TNF-α inhibitory action on PPARα gene expression. Taken together, these results demonstrate that TNF-α down-regulatory effect on PPARα expression is possibly signalled through the IKK/p105/p50/p65 pathway and phosphorylation of p65 at Ser468 and Ser536 plays a crucial role in the mechanism that limits PPARα production.

ACKNOWLEDGEMENT

Last but not least, my deepest gratitude goes to my beloved parents, brother, sister, and sister-in-law for their endless love, prayers, and encouragement. The amount of energy put into this project is enormous. This effort is most of the time only understood by those who also had gone through all the hardship and struggle in the laboratory. Hence, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project; your kindness means a lot to me. Thank you very much!

<div align="center">**APPROVAL SHEET**</div>

This thesis entitled "**STUDY ON THE TNF-α-STIMULATED NF-κB PATHWAY REGULATING PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARα) EXPRESSION IN HEPG2 CELLS"** was prepared by LIM WYI SIAN and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Assist. Prof. Dr. CHEW CHOY HOONG)   Date:…………………

Supervisor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

_____

(Assist. Prof. Dr. SAY YEE HOW)   Date:…………………

Co-supervisor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

Date: _____

SUBMISSION SHEET

It is hereby certified that **LIM WYI SIAN** (ID No: **09ADM06783**) has completed this thesis entitled **"STUDY ON THE TNF-α-STIMULATED NF-κB PATHWAY REGULATING PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARα) EXPRESSION IN HEPG2 CELLS"** under the supervision of Dr. Chew Choy Hoong (Supervisor) and Dr. Say Yee How (Co-supervisor) from the Department of Biomedical Science, Faculty of Science.

I understand that University will upload softcopy of my thesis in PDF format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____
LIM WYI SIAN

DECLARATION

I, Lim Wyi Sian hereby declare that the thesis 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 at UTAR or other institutions.

_____

LIM WYI SIAN

Date: _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_{230}	Absorbance at 230 nm wavelength
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AF	Activation function
Ala	Alanine
AP-1	Activator protein-1
Apo	Apolipoprotein
ARP	ApoAI regulatory protein
bp	Base pair
C/EBP	CCAAT-enhancer-binding protein
CREB	cyclic AMP response element-binding protein
CBP	CREB-binding protein
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
DBD	DNA binding domain
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Dnmt3	DNA methyltransferase
dNTPs	Deoxyribonucleoside triphosphates
DR	Direct repeat
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase
FADD	FAS-associated death domain
FBS	Fetal bovine serum
GABP	GA-binding protein
GSK3	Glycogen synthase kinase 3
HAT	histone acetyl transferase
HDAC	histone deacetylase
HDL	High-density lipoprotein
HepG2	Hepatocellular carcinoma
HNF4	Hepatic nuclear factor 4
IKK	IκB kinase
IL	Interleukin
IκB	Inhibitory protein of NF-κB
JNK	c-Jun N-terminal kinase
kb	Kilobase
kPa	Kilo pascal
LBD	Ligand binding domain
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblasts
MEK	Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
MEKK3	Mitogen-activated protein 3 kinase
MEM	Eagle's Minimum Essential Medium

mRNA	Messenger ribonucleic acid
MSK1	Mitogen- and stress-activated protein kinase-1
mTOR	mammalian target of rapamycin
NCoR	Nuclear-receptor corepressor complex
NEMO	NF-κB essential modulator
NF-AT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-kappa B
NIK	NF-κB-inducing kinase
PBS	Phosphate buffered saline
PGC-1	PPAR-gamma coactivator-1
PI3K	Phosphoinositide-3-kinase
PIAS1	Protein inhibitor of activated STAT1
PKA	Protein kinase A
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
SCF/ βTrCP	Skp1/Cullin/F-box/ beta-transducing repeat-containing protein
Ser	Serine
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SMRT	Silencing mediator for retinoid and thyroid hormone receptor
SRC	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
TAD	Transcriptional activation domain
Taq	*Thermus aquatics*
Thr	Threonine
T_m	Melting temperature
TNFAIP3	Tumour necrosis factor-alpha inducible protein 3
TNFR	Tumour necrosis factor-receptor
TNF-α	Tumour necrosis factor-alpha
TRADD	TNF-receptor-associated death domain
TRAF	TNF-receptor-associated factor
Ubc	ubiquitin-conjugating enzyme
v/v	Volume/volume
w/v	Weight/volume
β-actin	Beta-actin

CHAPTER 1

INTRODUCTION

Liver is the central regulator in regulating metabolism and a response to exogenous stimuli. Every form of liver disease is accompanied by some degree of inflammation (Dasarathy, 2008). Cells in the liver express various recognition receptors that sense endogenous or pathogen-derived signals and induce the specific intracellular signal transduction pathways to produce inflammatory mediators and interferons (Szabo *et al.*, 2006; Mandrekar and Szabo, 2011). Immune cells as well as parenchymal cells in the liver produce various chemokines and cytokines that through cross-talk between these cell populations affect the outcome and prognosis of the liver inflammation (Mandrekar and Szabo, 2011). Furthermore, inflammation has long been associated with the development of cancer. Epidemiological studies have established that many tumours occur in association with chronic infectious disease (Coussens and Werb, 2002; Rakoff-Nahoum, 2006; Berasain *et al.*, 2009). It is also known that persistent inflammation increases the risk and accelerates the development of cancer, such as hepatocellular carcinoma (HCC). HCC is the major form of primary liver cancer which is one of the most deadly human cancers (He and Karin, 2011).

Tumour necrosis factor-alpha (TNF-α) is one of the key inflammatory cytokines which is best known for its role in the liver's acute phase response. TNF-α exerts many of its functions via activation of nuclear

factor-kappa B (NF-κB), the ubiquitous inducible transcription factor found to promote hepatocyte survival in both developing and adult livers (He and Karin, 2011). In addition, it also plays a crucial role in liver inflammatory responses by controlling the expression of an array of growth factors and cytokines (Palomer *et al.*, 2009; He and Karin, 2011). Inflammatory responses driven by the increase in TNF-α are known to be related to the pathology of metabolic disorders. A gene transcription profiling was examined in control and TNF-α treated HepG2 cells which indicated that TNF-α significantly altered the transcriptome profiling within HepG2 cells with genes involved in lipid and steroid metabolism (Pandey *et al.*, 2010).

The expression of the metabolic genes is controlled at least in part by the peroxisome proliferator-activated receptor (PPAR) transcription factors family. There are three different PPAR subtypes, PPARα, PPARβ/δ, and PPARγ (Issemann and Green, 1990; Dreyer *et al.*, 1992; Kliewer *et al.*, 1994). PPARα is a nuclear hormone receptor involved in regulation of fatty acid oxidation and transport, which requires heterodimerisation with another nuclear receptor, the retinoid X receptor (RXR), in order to be activated (Palomer *et al.*, 2009). The heterodimerisation is then followed by coactivator recruitment and binds to DNA at peroxisome proliferator response elements (PPRE), resulting in target gene transcription (Bardot *et al.*, 1993; Palomer *et al.*, 2009). PPARα is most prominently expressed in hepatocytes, muscle and heart myocytes, pancreas and kidney and can be activated by fatty acids (FA), eicosanoids, and fibrates (Chinetti *et al.*, 2001; Shearer and Hoekstra, 2003; Faiola *et al.*, 2008). Activation of PPARα results in the increase uptake and

oxidation of free FA through mitochondrial and peroxisomal β-oxidation (Reddy and Hashimoto, 2001; Hansmannel *et al.*, 2003). Several experimental animal and cellular observation indicate that PPARα activation prevents hepatic triacylglycerol (TAG) infiltration under conditions of increased hepatic FA influx or decreased hepatic FA efflux by increasing the rate of fatty acid catabolism (Kersten *et al.*, 1999; Hashimoto *et al.*, 2000; Harano *et al.*, 2006). Moreover, several studies have shown that PPARα also modulates the inflammatory response (Forman *et al.*, 1997; Ip *et al.*, 2004; Lefebvre *et al.*, 2006). These data taken together, indicates an intriguing possibility that PPARα is an important transcription factor which is responsible in the mechanism of coordinating the regulation of multiple genes that are central to the cause of most of the liver diseases. Understanding the molecular signalling pathways mediating these processes is important for the identification of novel therapeutic targets for these diseases.

Thus, human hepatocellular carcinoma cell line (HepG2) was used as a model system to elucidate the expression profile of PPARα. The objectives of this study were:

i)   To investigate the dose and time-course response of PPARα expression upon TNF-α stimulation;

ii) To determine the effect of TNF-α on PPARα mRNA stability;

iii) To investigate the effects of NF-κB inhibition on TNF-α-stimulated PPARα and its target genes expression, such as ApoAI and ApoB;

iv)   To delineate the upstream signalling events focusing on the NF-κB pathway which leads to the reduction of PPARα gene expression in HepG2 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are members of the steroid hormone nuclear receptor superfamily of ligand-dependent transcription factors. They play an important role in the regulation of lipid and lipoprotein metabolism, energy homeostasis, inflammation, artherosclerosis and glucose homeostasis (Devchand *et al.*, 1996; Staels *et al.*, 1998a; Evans *et al.*, 2004; Berger *et al.*, 2005; Yang *et al.*, 2008). There are three isoforms of PPAR that have been identified and cloned, which includes PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3) (Issemann and Green, 1990; Dreyer *et al.*, 1992; Kliewer *et al.*, 1994). All three isoforms exhibit distinct tissue distributions, as well as different ligand specificities reflecting their biological functions (Desvergne and Wahli, 1999; Willson *et al.*, 2001; Yang *et al.*, 2008). In addition, all PPAR isoforms appear to play important roles in inflammation and immunity (Chinetti *et al.*, 2000; Daynes and Jones, 2002; Pang *et al.*, 2003; Sampath and Ntambi, 2004; Stienstra *et al.*, 2007).

PPAR isoforms can be activated by polysaturated fatty acids and naturally occurring fatty acid-derived molecules (Kliewer *et al.*, 1994; Braissant *et al.*, 1996; Desvergne and Wahli, 1999; Zomer *et al.*, 2000). It has been difficult to definitively establish the exact molecular species of fatty acids and/or their metabolites that bind to the various PPARs *in vivo*. However,

considerable circumstantial evidence suggests that PPARs function as sensors of a variety of molecules that are derived either from extracellular or intracellular fatty acid metabolism (Dreyer *et al.*, 1993; Chawla *et al.*, 2003; Ricote and Glass, 2007). In addition, PPARs can be regulated by several synthetic compounds, such as hypolipidemic drugs and synthetic antidiabetic thiazolidinedione (TZD) which are well known ligands for PPARα and PPARγ, respectively (Keller *et al.*, 1997; Zierath *et al.*, 1998).

2.2 PPARα

PPARα was the first member of the PPAR family to be cloned from murine species and it was named based on its ability to be activated by peroxisome proliferator chemicals (Issemann and Green, 1990). It has subsequently been cloned from frog (Dreyer *et al.*, 1992), rat (Göttlicher *et al.*, 1992), human (Sher *et al.*, 1993), and rabbit (Guan *et al.*, 1997). Human PPARα has been mapped to chromosome 22 adjacent to the region 22q12-q13.1 (Sher *et al.*, 1993). It contains an approximately 70 amino acid DNA-binding domain comprised of two highly conserved zinc fingers and a C-terminal ligand-binding domain (LBD) of approximately 250 amino acids (Moras and Gronemeyer, 1998; Sertznig *et al.*, 2007).

PPARα is a ligand-activated transcription factor that controls expression of a variety of genes related to fatty acid transport across the cell membrane, intracellular lipid trafficking, mitochondrial and peroxisomal fatty acid uptake, and both mitochondrial and peroxisomal fatty acid β-oxidation (Desvergne and Wahli, 1999; Francis *et al.*, 2003; Mandard *et al.*, 2004).

Studies using PPARα-null mice by Lee *et al.* (1995) and PPARα ligands by Desvergne *et al.* (2004) revealed a physiological role for PPARα in stimulation of fatty acid catabolism under conditions of fasting. It is known to be preferentially expressed in the liver (Schoonjans *et al.*, 1996; Chinetti *et al.*, 2000; Reddy and Hashimoto, 2001), and in tissues with high rates of metabolism and fatty acid catabolism, such as the kidney, skeletal muscle, heart and brown fat (Auboeuf *et al.*, 1997; Gonzalez, 1997; Chinetti *et al.*, 2000; Berger and Moller, 2002). It is also expressed in vascular cells such as endothelial cells (Inoue *et al.*, 1998), vascular smooth muscle cells (VSMC) (Staels *et al.*, 1998b; Diep *et al.*, 2000) and monocytes/macrophages (Chinetti *et al.*, 1998). Activation of PPARα also appears to play a role in cellular differentiation, including promotion of keratinocyte differentiation and wound-healing in skin (Hanley *et al.*, 1998; Hanley *et al.*, 2000; Michalik *et al.*, 2001; Schmuth *et al.*, 2004).

2.2.1   Structure overview

PPARα shares common structural characteristics with other members of nuclear receptor family. There are four functional domains that have been identified, called A/B, C, D, and E/F (Figure 2.1a). The N-terminal A/B domain contains a ligand independent activation function 1 (AF-1) responsible for the phosphorylation of PPARα and has a low level of basal transcriptional activity (Werman *et al.*, 1997; Desvergne and Wahli, 1999). The DNA binding domain (DBD) or C domain contains 70 amino acids and forms two zinc fingers motifs which promote the binding of PPARα to the PPRE in the

(a)

(b)
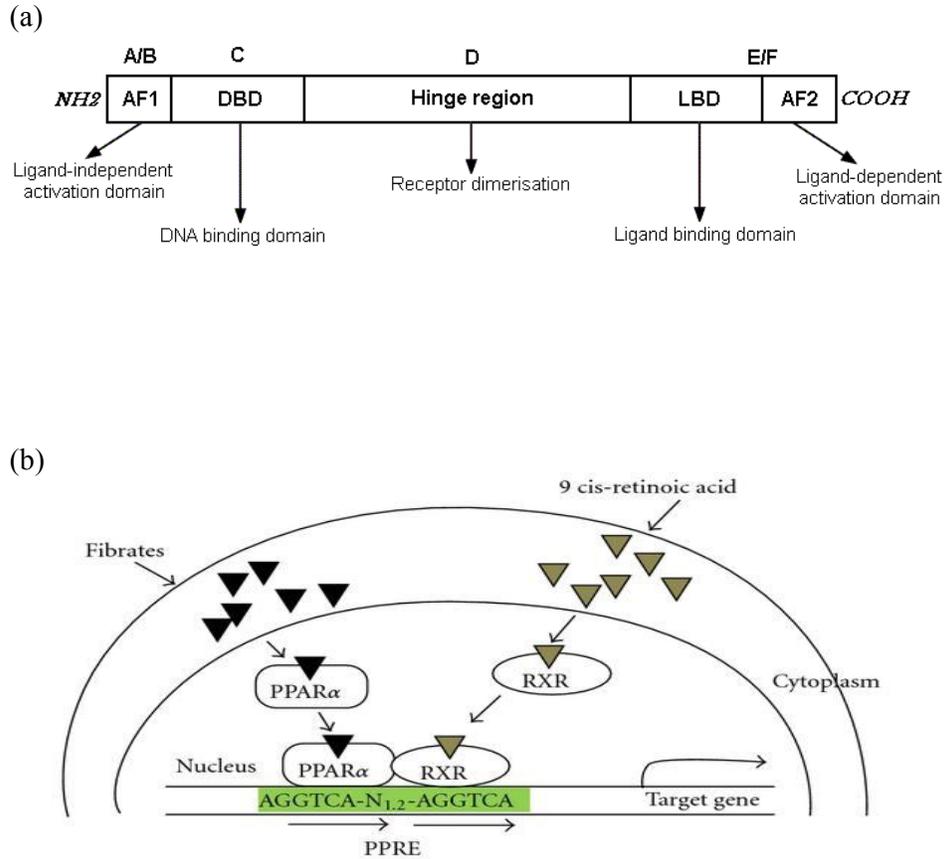
Figure 2.1: Domain structure and activation of PPARα.
(a) PPARα contain the following functional regions: a N-terminal domain with AF-1 domain (ligand-independent activation domain), a DNA binding domain (DBD) with two zinc fingers, a hinge region, and a C-terminal ligand binding domain (LBD) and AF-2 domain (ligand-dependent activation domain). (b) PPARα functions as heterodimers with retinoid X receptors (RXRs) and is activated by specific ligands. The heterodimers then modulate DNA transcription by binding to the promoter region of target genes, termed PPRE, which are composed of 2 degenerate hexanucleotide repeats (arrows) arranged in tandem as direct repeats spaced by 1 nucleotide (Adapted from Staels *et al.*, 1998a; Chan *et al.*, 2010).

promoter region of target genes (Kliewer *et al.*, 1992; Moras and Gronemeyer, 1998). The D site is a docking domain for cofactors. This hinge region also binds corepressor proteins, with the characteristic LXXXIXXXL (L, leucine; I, Isoleucine; X, any amino acid) repressor motif to the receptor in its quiescent, unliganded state. The corepressor-binding site partly overlaps with the coactivator-binding site (Dowell *et al.*, 1999). It influences intracellular trafficking and subcellular distribution. The C-terminal E/F domain or ligand binding domain (LBD) has an extensive secondary structure consisting of 13 alpha helices and a beta sheet (Desvergne and Wahli, 1999; Zoete *et al.*, 2007). It is responsible for ligand specificity and PPARα activation for the binding to the PPRE, which would in turn increase the expression of targeted genes (Berger and Moller, 2002). Following ligand-binding, the AF-2 domain undergoes a conformational change in the protein which allows interaction of the receptor with the LXXLL motifs located in coactivator proteins (Xu *et al.*, 2001). The recruitment of PPARα cofactors assists the gene transcription processes.

Like other members of the nuclear receptor gene family, all PPAR subtypes possess a common domain structure which contains DBD and LBD. Willson and colleagues (2000) showed that PPAR subtypes are highly conserved in DBD indicating that they share similar DNA binding site present on promoter region of the target genes; while the LBD have slightly lower level of conservation across the subtypes, suggesting that the PPAR subtypes are ligand-specific. The N-terminal domain of the receptor shows low

sequence identity across the subtypes and is responsible for differences in the biological function of the subtypes (Castillo *et al.*, 1999).

2.2.2   Activation and modulation of PPARα

Nuclear receptors can be activated in ligand-dependent and ligand-independent mechanisms. The canonical pathway by which PPARα modulate the expression of target genes involves their activation by ligand binding. Physiological ligands of PPARα include fatty acids and their derivatives. PPARα requires heterodimerisation with retinoid X receptor (RXR) (NR2B), the receptor for 9-*cis* retinoic acid (9-*cis* RA), and bind to specific response elements termed peroxisome proliferator response elements (PPREs) in target gene promoters to initiate transcriptional activity (Kliewer *et al.*, 1992; Tan *et al.*, 2005) (Figure 2.1b). Structurally, PPREs consist of direct repeat (DR)-1 or DR-2 elements of two hexanucleotides with the AGGTCA sequence separated by a single or two nucleotides spacer (Kliewer *et al.*, 1992; Lemberger *et al.*, 1996). In general, this sequence occurs in the promoter region of a gene involved in carbohydrate and lipid metabolism and is specific for PPAR-RXR heterodimer. When the PPARα binds to its ligand, it modulates transcription of target gene by recruiting a number of coactivators or corepressors by increasing or decreasing, depending on the gene (Gearing *et al.*, 1993; Yu and Reddy, 2007).

Besides this classical ligand-dependent control, several studies have reported the modulation of PPARα activity by phoshorylation (Shalev *et al.*, 1996; Lazennec *et al.*, 2000; Gelman *et al.*, 2005). PPARα is a phosphoprotein

and its activity can be modulated by its phosphorylation status (Shalev *et al.*, 1996). Phosphorylation affecting the A/B domain of the receptor can modulate its ligand-independent AF-1 transactivating function (Werman *et al.*, 1997; Hi *et al.*, 1999). Transfection experiments have suggested that phosphorylation on the A/B domain induces a dissociation of corepressors such as nuclear-receptor corepressor complex (NCoR) from C- terminus of PPARα, thereby relieving their inhibitory action on transcription (Dowell *et al.*, 1999). Along the same lines, phosphorylation of the A/B domain of PPARα enhances coactivation by the PPARγ coactivator-1 (PGC-1), which interacts with the E domain of PPARα (Vega *et al.*, 2000; Barger *et al.*, 2001). Very interestingly, this phosphorylation-induced cooperation did not extend to other coactivators such as steroid receptor coactivator-1 (SRC-1) and the PPAR-binding protein (PBP), suggesting that phosphorylation in the A/B domain may dictate coactivator selectivity (Gelman *et al.*, 2005).

Furthermore, phosphorylation also affects the interaction between PPARα and DNA. For instance, the phosphorylation of PPARα by protein kinase A (PKA) on the C domain increases DNA binding in the absence and the presence of exogenous ligands (Lazennec *et al.*, 2000). In addition, *in vitro* kinase assays reveal that PPARα is also phosphorylated by glycogen synthase kinase 3 (GSK3) predominately at serine 73 in the A/B domain (Burns, 2007). This decreased the stability of PPARα via degradation of the ubiquitin proteasome system. Phosphorylation of PPARα upon p38 kinase activation enhances the transcriptional activity of this nuclear receptor by increasing its interaction with the transcriptional coactivator PGC-1, which then favours

cardiac fatty acid β-oxidation during periods of stress (Barger *et al.*, 2000; Barger *et al.*, 2001). Interestingly, mitogen-activated protein kinase (MAPK) pathways are also activated during myocyte hypertrophic growth and, in this model, the extracellular signal-regulated kinase (ERK)-mediated phosphorylation of PPARα was shown to have an opposite effect (i.e. inhibition) (Barger *et al.*, 2000) to that described above for the p38 kinase. On the other hand, inhibition of protein kinase C (PKC) had a dual effect on PPARα. PKC inhibition decreased transactivation capacity of PPARα, but enhanced its transrepression activity (Blanquart *et al.*, 2004). The molecular mechanisms underlying these differences are not known, but this exemplifies the very context-specific biological outcome of nuclear receptor phosphorylation.

2.2.3   Ligands of PPARα

The ligand-binding domain of PPARα is unusually large, and as consequence, they are relatively promiscuous in a sense that they are activated by a number of natural and synthetic ligands of different chemical structure (Table 2.1) to increase the transcription activity.

As reviewed by Hihi *et al.* (2002), PPARα is activated by a variety of long-chain fatty acids, in particular by polysaturated fatty acids such as palmitic acid, oleic acid, linoleic acid, and arachidonic acid. The beneficial effects of PPARα ligands in variety of diseases have been established. The hypolipidemic fibrates including gemfibrozil, fenofibrate, and bezafibrate, are a class of drugs widely prescribed for reducing triglyceride levels, a risk factor

Table 2.1   Natural and synthetic ligands for PPARα.

Categories	Ligands	References
Exogenous (Synthetic) ligands	Wy-14643	Keller *et al.*, 1997
	Clofibrate	Willson *et al.*, 2000
	Fenofibrate	Schoonjans *et al.*, 1996
	Bezafibrate	Bradford *et al.*, 1992
	Ibuprofen	Lehmann *et al.*, 1997
	Indomethacin	Lehmann *et al.*, 1997
	Fenoprofen	Lehmann *et al.*, 1997
	SB219994	White *et al.*, 2003
	GW2331	Kliewer *et al.*, 1997
	GW2433	Brown *et al.*, 1997
	GW7647	Brown *et al.*, 2001
	GW9578	Brown *et al.*, 1999;
	GW409544	Xu *et al.*, 2001
Endogenous (Natural) ligands	8(S)-hydroxyeicosatetraenoic acid (HETE)	Yu *et al.*, 1995
	Oleic acid	Sterchele *et al.*, 1996;
	Leukotriene B4 (LTB4)	Devchand *et al.*, 1996
	Palmitic acid	Michaud and Renier, 2001
	Linoleic acid	Göttlicher *et al.*, 1992
	Arachidonic acid	Xu *et al.*, 1999
	Oleoylethanolamide	Fu *et al.*, 2003

for cardiovascular disease. Fibrates increase levels of high-density lipoprotein (HDL), reduce levels of low-density lipoprotein (LDL), and slow down the progression of premature coronary atherosclerosis, particularly in patients with type 2 diabetes (Chait *et al.*, 1993; Vu-Dac *et al.*, 1995; Evans *et al.*, 2000; Rubins *et al.*, 2002; Ansquer *et al.*, 2005; Calkin *et al.*, 2006; Sertznig *et al.*, 2007; Slim *et al.*, 2011). Moreover, Park *et al.* (2006) who demonstrate that fenofibrate reduces fasting blood glucose, ameliorates insulin resistance, reduces hypertrophy of pancreatic islets, and reduces urinary albumin excretion, had implicated a potential therapeutic target for PPARα agonists in treating type 2 diabetes and its renal complications. Another compound known as ureidofibrates, such as GW2331 and GW9578, have been reported as potent and subtype-selective PPARα agonists with lipid lowering activity (Brown *et al.*, 1999). However, fibrates are weak agonists of PPARα, and high doses are required for effective treatment (Brown *et al.*, 1999).

In addition, the development of dual PPARα/γ agonists (glitazars) and pan-PPAR agonists (GW766954, GW625019, and PLX-204) have been shown to improve glycaemic and lipid metabolism in a range of settings (Chakrabarti *et al.*, 2003; Chang *et al.*, 2007; Mittra *et al.*, 2007). Interestingly, certain non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin, fenoprofen, flufenamic, and ibuprofen, have been shown to bind and activate PPARα at high micromolar concentrations, with fenoprofen activating the receptor to a degree comparable to that obtained with Wy-14643 (Lehmann *et al.*, 1997; Keller *et al.*, 1997).

2.3   Transcriptional activities of PPARα

2.3.1   Ligand-dependent transactivation

In the absence of ligand, PPARα maintain an association with corepressor complexes such as NCoR and the related silencing mediator for retinoid and thyroid hormone receptor (SMRT), and can be stabilised by antagonists (Chen and Evans, 1995; Dowell *et al.*, 1999; Jepsen *et al.*, 2000). Upon binding to their ligands, PPARα undergoes conformational changes that allow corepressor release and coactivator complexes recruitment which modify chromatin structure (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). Subsequently, the prototypic activity of PPARα is to activate transcription in a ligand-dependent manner following direct binding to DNA response elements in the promoter or enhancer region of target genes (so called DR-1 elements or PPREs). This is known as ligand-dependent transactivation (Figure 2.2a). For example, the binding of fibrates (Section 2.2.3) to PPARα induces the activation of multiple genes involved in lipid metabolism through the binding of the activated PPARα to PPRE located in the gene promoters.

2.3.2   Ligand-dependent transrepression

PPARα ligand exert its anti-inflammatory effects by interference with signal-dependent activation of various transcription factors, such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), CCAAT-enhancer-binding protein (C/EBP), signal transducer and activator of transcription (STAT), and nuclear factor of activated T-cells (NF-AT) (Delerive *et al.*, 1999a; Marx *et al.*, 1999; Delerive *et al.*, 2001; Gervois *et al.*, 2004; Planavila

15
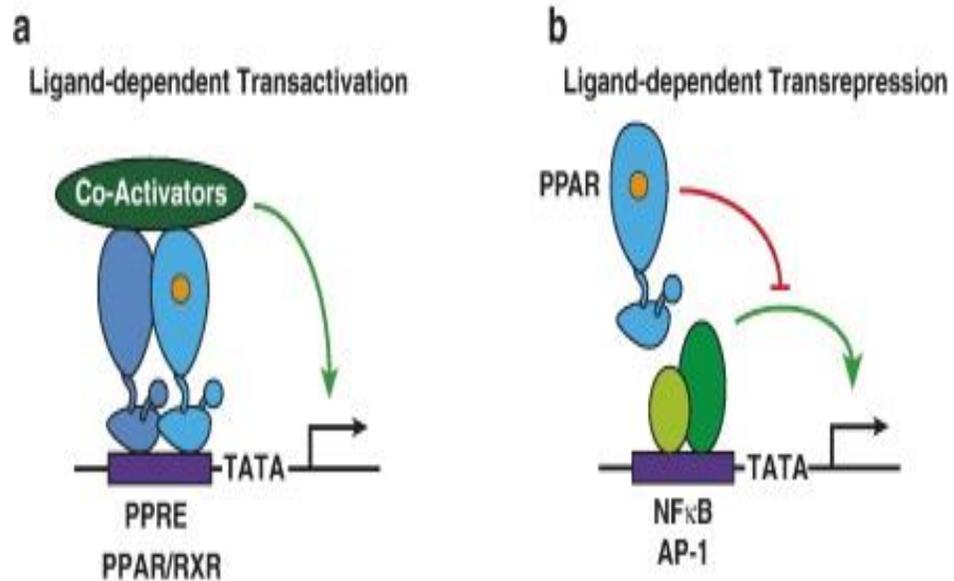
Figure 2.2: Transcriptional activities of the PPARα.
(a) Ligand-dependent transactivation. PPARα activates transcription in a ligand-dependent manner by binding directly to specific PPRE in target gene promoter as heterodimers with RXR. Binding of agonist ligand leads to the recruitment of coactivator complexes that modify chromatin structure and facilitate assembly of the general transcriptional machinery to the promoter. (b) Ligand-dependent transrepression. PPARα represses transcription in a ligand-dependent manner by antagonising the actions of other transcription factors, such as NF-κB and AP-1. (Adapted from Ricote and Glass, 2007).

et al., 2005a). In contrast to transcriptional activation, transrepression itself represents a molecular mechanism by which PPARα can inhibit signal-dependent gene activation without direct, sequence-specific binding to DNA (Glass and Ogawa, 2006, Pascual and Glass, 2006) (Figure 2.2b). Several different models of transrepression have been proposed. However, it has been difficult to identify a unified mechanism of repression by activated PPARα. It is possible that there are mechanisms specific to the signal, cell type, and even the gene promoter.

In the direct interaction model, PPARα can transrepress non-PPREs containing genes by protein-protein interactions with these transcription factors and prevent them from binding to their response elements (Figure 2.3a). Ligand-activated PPARα has been shown to inhibit the vascular inflammatory response by direct protein-protein interaction with p65, c-Jun, and CBP in smooth muscle cells (Delerive *et al.*, 1999a; Delerive *et al.*, 2002). However, PPARα activators do not inhibit all NF-κB-driven target genes and their effect is promoter-context dependent (Delerive *et al.*, 2002). In a similar manner, PPARα ligands interfere with the AP-1 signalling pathway, which mediates thrombin-activation of endothelin-1 (ET-1) gene expression in endothelial cells (Delerive *et al.*, 1999b).

In addition, PPARα ligands in smooth muscle cells and hepatocytes induce the expression of inhibitory protein IκBα, leading to retention of the NF-κB subunits in the cytoplasm and consequently suppress their DNA binding activity (Delerive *et al.*, 2000, Vanden Berghe *et al.*, 2003)
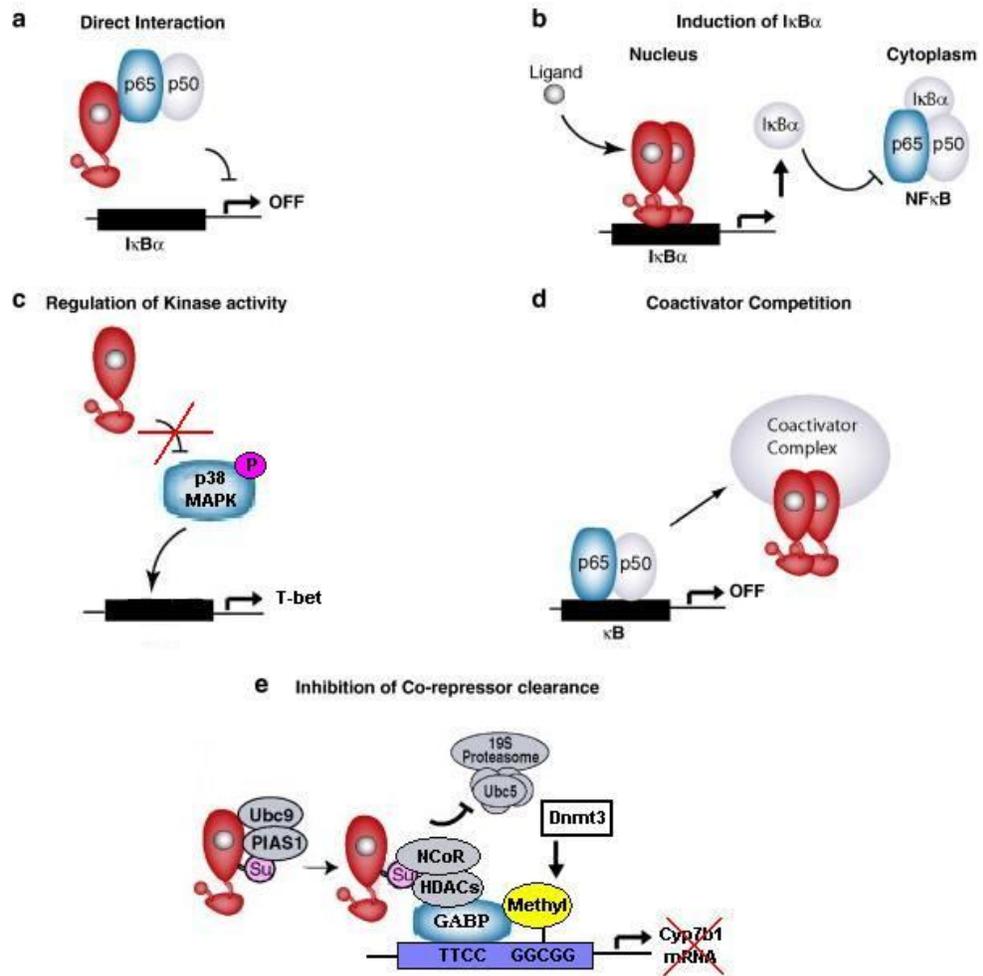
Figure 2.3: Mechanisms of PPARα-mediated transrepression.
(a) Direct interaction between PPARα and p65 subunit. (b) Induction of IκBα expression. (c) Activation of PPARα inhibits JNK/MAPK activity. (d) Competition for a limiting pool of coactivators, such as CREB-binding protein. (e) Corepressor-dependent model of transrepression. AP-1, activator protein-1; NCoR, nuclear-receptor corepressor complexes; HDAC, histone deacetylase; GABP, GA-binding protein; Dnmt3, DNA methyltransferase 3; Ubc9, ubiquitin-conjugating enzyme; PIAS1, protein inhibitor of activated STAT 1 (Adapted from Ricote and Glass, 2007; Leuenberger *et al.*, 2009).

(Figure 2.3b). Similarly, fibrates down-regulate IL-1β-induced C-reactive protein (CRP) expression in PPARα-dependent manner, via reduction of the formation of the nuclear p50/C/EBPβ complex due to an increase in IκBα expression and to a decrease in the hepatic p50 and C/EBPβ protein levels (Kleemann *et al.*, 2003, Kleemann *et al.*, 2004).

Previous studies also have provided evidence that PPARα can regulate kinase activity at different levels (Figure 2.3c). Researchers have shown that PPARα ligands inhibit endothelial cell migration by inhibition of vascular endothelial growth factor (VEGF)-induced Akt phosphorylation (Goetze *et al.*, 2002). Furthermore, it has been shown that the anti-inflammatory effect of PPARα in macrophages of the inhibition of the PKC signalling pathway (Blanquart *et al.*, 2004). Jones and colleagues (2003) have shown that unliganded PPARα suppressed the phosphorylation of p38 MAPK after CD4+ T-cell stimulation. In contrast, PPARα ligand treatment led to the relaxation of this suppression effect and consequent increased p38 MAPK phosphorylation and promoted T-bet expression which is important in the inhibition and termination of activation-induced cytokine gene transcription (Szabo *et al.*, 2000). This proves a contradictory role of PPARα in p38 MAPK signalling.

PPARα and many of the transcription factors that drive inflammatory responses require an overlapping set of coactivator proteins. In the coactivator competition model, PPARα compete with NF-κB and AP-1 for binding to the general coactivators CBP and p300, or other coregulators, which are present in

the cell in limiting amounts (Kamei *et al.*, 1996; Ricote and Glass, 2007; Yu and Reddy, 2007) (Figure 2.3d). Gervois *et al.* (2001) have showed that titration of SRC-2 by activated PPARα will negatively interfere with C/EBPβ. Sequestration of coactivators, including SRC-1 and SRC-3/pCAF, has been shown to dramatically impair the transcriptional activity of NF-κB (Sheppard *et al.*, 1999). Studies by Kane and co-workers (2009) suggested that piperidine, a PPARα agonist, is a strong recruiter of SRC-1 and PGC-1α, and additional coactivators, including SRC-2, SRC-3, and PGC-1β. Therefore, PPARα may transrepress NF-κB, C/EPBβ, and potentially other inflammatory mediators in part by coactivator competition.

De Bosscher *et al.* (2000) have however demonstrated that transrepression still occurs in the presence of excess coactivators. Although initially identified as corepressors of nuclear receptors, subsequent studies demonstrated that NCoR and SMRT function as corepressors required for active repression of genes under transcriptional control of numerous additional signal-dependent transcription factors, including NF-κB and AP-1 (Hoberg *et al.*, 2004; Ogawa *et al.*, 2004). Recent studies have led to another model of corepressor-dependent mechanism which is a female-specific PPARα-dependent transrepression (Leuenberger *et al.*, 2009) (Figure 2.3e). Using the steroid oxysterol 7α-hydroxylase cytochrome P450 7b1 (*Cyp7b1*) gene as a model, ligand-dependent SUMOylation of the PPARα LBD triggered the interaction of PPARα with the LXXLL motif of GA-binding protein α (GABPα) bound to the target *Cyp7b1* promoter. Leuenberger *et al.* (2009) have observed that SUMOylated PPARα is more abundant in female mice

20

compared to male mice. After SUMOylation, corepressor NCoR, HDACs, histone, and DNA methyltransferases were recruited (Pourcet *et al.*, 2010), and the adjacent Sp1-binding site and histones were methylated. This mechanism hinders Sp1 from binding to its methylated site thus mediating down-regulation of *Cyp7b1* (Wu and Chiang, 2001). The repression of *Cyp7b1* by PPARα suggested a possible protective effect of PPARα in estrogen-related liver diseases, including inflammation and cholestasis (Schreiber and Simon, 1983; Tang *et al.*, 2006; Shi *et al.*, 2010).

2.4   Signalling mechanisms by tumour necrosis factor-alpha (TNF-α)

TNF-α is a pleiotropic cytokine which has been demonstrated to regulate and interfere with energy metabolism, especially lipid homeostasis (Grunfeld *et al.*, 1996; Tacer *et al.*, 2007; Chen *et al.*, 2009). Thus, it is a mediator of hepatic injury by activating several intracellular pathways to regulate inflammation, cell death, and proliferation (Schwabe and Brenner, 2006, Wullaert *et al.*, 2007). The biological responses to TNF-α are mediated through two structurally distinct receptors: the 60 kDa TNF receptor 1 (TNFR1) and the 80 kDa TNFR2 (Hsu *et al.*, 1995, Sawanobori *et al.*, 2003) (Figure 2.4). Both receptors are ubiquitously expressed transmembrane glycoproteins that trimerise upon ligand binding. Although TNFR1 and TNFR2 are highly homologous in their ligand-binding extracellular domains, their intracellular domain exhibit no sequence homology and they activate divergent signalling pathways (Locksley *et al.*, 2001; MacEwan, 2002).
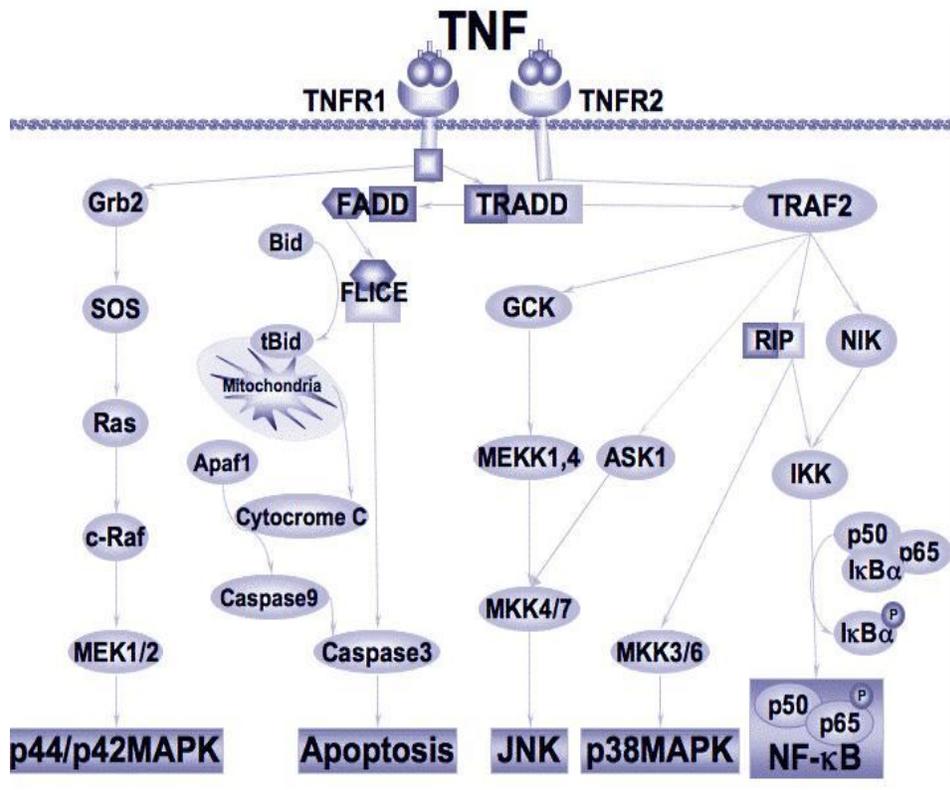
Figure 2.4: The downstream signalling pathways of TNF-α.
The TNF-α can activate several signal transduction pathways to induce apoptosis, cell survival or inflammation acting via two receptors: TNFR1 and TNFR2. TNF-α induces apoptosis by binding caspase-8 (FLICE) to FADD and promotes inflammation and survival, which is mediated through TRAF2 via JNK-dependent kinase cascade, MEKK kinase cascade, and NF-κB activation by RIP (Adapted from Sethi *et al.*, 2008).

The cytoplasmic domain of the TNFR1 has a death domain, which has been shown to sequentially recruit TNF-receptor associated death domain (TRADD), FAS-associated death domain (FADD), and caspase-8 (also called FLICE), leading to caspase-3 activation, which in turn induces apoptosis by causing degradation of multiple proteins (Nagata and Golstein, 1995). TRADD also recruits TNF-receptor-associated factor 2 (TRAF2), which through receptor-interacting protein (RIP) activates IKK leading to IκBα phosphorylation, ubiquitination and degradation. This would then lead to NF-κB activation. Through recruitment of TRAF2, TNF activates various MAPK, including the c-Jun N-terminal kinase (JNK), p38 MAPK, and p42/p44 MAPK. Meanwhile, these protein kinase cascades were also reported to affect the expression or transcriptional activity of PPARα (Barger *et al.*, 2000; Irukayama-Tomobe *et al.*, 2004; Diradourian *et al.*, 2008; Drosatos *et al.*, 2011). TRAF2 is also essential for the TNF-induced activation of Akt, another cell-survival signalling pathway. Thus, TNFR1 activates both apoptosis and cell survival signalling pathways simultaneously (Darnay and Aggarwal, 1997; Aggarwal and Takada, 2005).

In contrast to TNFR1, since TNFR2 lacks a cytoplasmic death domain, it can interacts directly with TRAF2 (Rothe *et al.*, 1994) and mediates TNF-induced apoptosis (Haridas *et al.*, 1998). Gene-deletion studies have shown that TNFR2 can also activate NF-κB, JNK, p38 MAPK, and p42/p44 MAPK (Mukhopadhyay *et al.*, 2001). Thus, both TNFR1 and TNFR2 can activate NF-κB via the indirect and direct recruitment of the TRAF2 protein, respectively.

2.5   NF-*κ*B signalling pathway

NF-*κ*B is a group of structurally related transcriptional proteins that form homodimers or heterodimers composed of various combinations of members of the mammalian NF-*κ*B transcription factor family, which are NF-*κ*B1 (p50, its precursor p105), NF-*κ*B2 (p52, its precursor p100), p65 (RelA), RelB, and c-Rel (Lenardo and Baltimore, 1989; Baeuerle, 1991). A genetic study using a panel of knockout cell lines deficient in one or two NF-*κ*B protein genes did indeed reveal that different NF-*κ*B-regulated genes have distinct requirements for NF-*κ*B proteins (Hoffmann *et al.*, 2003). However, different forms of the NF-*κ*B dimer exhibit distinct properties in terms of DNA binding preference selectively of interaction with I*κ*B isoforms, and transcriptional capability. NF-*κ*B transcriptional activity is silenced by interactions with I*κ*B proteins through three mechanisms, by sequestrating NF-*κ*B dimers in the cytoplasm, facilitating dissociation of DNA-bound NF-*κ*B dimers from their DNA binding sites, and exporting NF-*κ*B dimers from nucleus (Liu and Malik, 2006). Thus, NF-*κ*B is present as a latent, I*κ*B-bound complex in the cytoplasm in most cells.

There are two signalling pathways leading to the activation of NF-*κ*B known as the canonical pathway (or classical) and the non-canonical pathway (or alternative) (Karin, 1999; Gilmore, 2006; Tergaonkar, 2006; Bhatnagar *et al.*, 2010) (Figure 2.5). Bhatnagar *et al.* (2010) has shown that the ability of TNF-α augments the activity of both classical and alternative NF-*κ*B signalling pathways. Although they are often activated concurrently, the two NF-*κ*B activation pathways converge at the IKK complex (Liu and Malik,
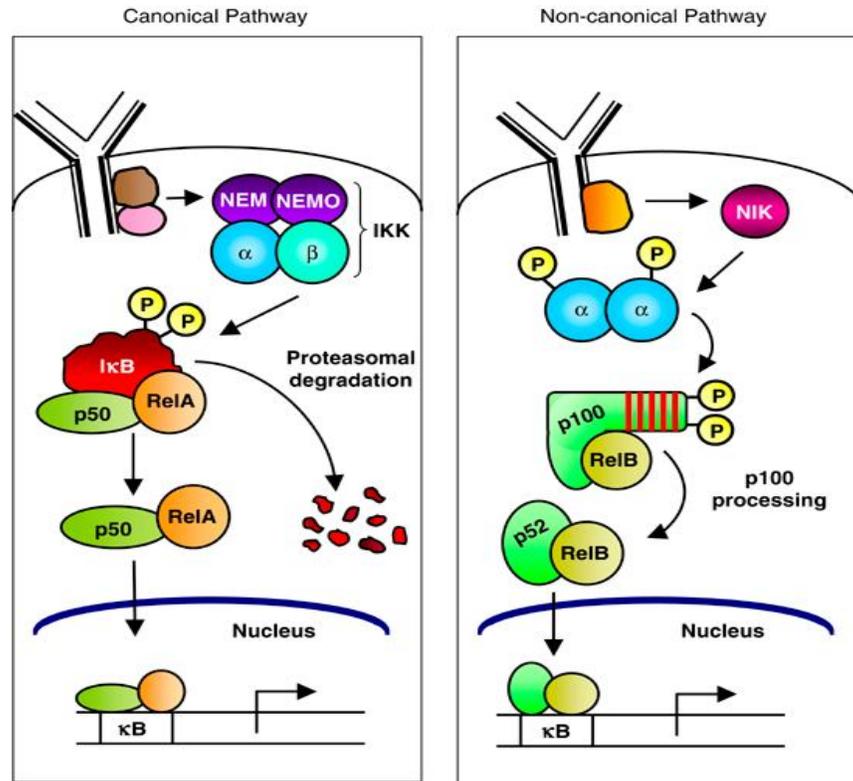
Figure 2.5: NF-κB signal transduction pathways.
In the canonical NF-κB pathway, NF-κB dimers such as p50/RelA are maintained in the cytoplasm by interaction with IκB molecule. In many cases, the binding of a ligand to a cell surface receptor (e.g. TNF-α) recruits adaptors (e.g. TRAFs and RIP) to the cytoplasmic domain of the receptor. In turn, these adaptors often recruit and activate the IKK complex (containing IKKα, β, and γ). IKK then phosphorylates IκB and leads to its ubiquitination and degradation by the proteasome. NF-κB then enters the nucleus to turn on target genes. By contrast, the non-canonical NF-κB activation pathway involves activating the NIK to stimulate IKKα-induced phosphorylation and proteolytic processing of p100. Activated p52 then forms a complex with RelB to translocate into the nucleus, thereby activating a distinct set of genes (Adapted from Gilmore, 2006).

2006; Israël, 2010) and have distinct regulatory functions (Silverman and Maniatis, 2001; Hayden and Ghosh, 2004).

In the canonical pathway, binding of ligand to a cell surface receptor such as TNFR leads to the recruitment adaptors (such as TRAF) to the cytoplasmic domain of the receptor. These adaptors in turn recruit the IKK complex consisting of catalytic kinase subunits (IKKα and/or IKKβ) and the regulatory non-enzymatic scaffold protein NEMO (also known as IKKγ) (Gilmore, 2006). The activated IKK phosphorylates the IκB proteins leading to their proteasomal degradation. IκB degradation leads the translocation of NF-κB dimer (comprising mainly of RelA, c-Rel, and p50) into the nucleus, where it binds to its consensus sequence on the promoter or enhancer regions of NF-κB-regulated genes, resulting in gene transcription (Liu and Malik, 2006; Demchenko *et al.*, 2010). In most cases, the activation of NF-κB is transient and cyclical in the presence of continual inducer. For instance, in mouse fibroblasts maintained in the presence of TNF-α, nuclear NF-κB DNA-binding activity appears and disappears approximately every 30 – 60 min; these cycles are due to repeated degradation and re-synthesis of IκB and the consequence activation and inactivation of NF-κB, respectively (Hoffmann *et al.*, 2006). Activation of canonical NF-κB is required for a successful immune response and to amplify the survival and proliferation of cells (Demchenko *et al.*, 2010).

In the alternative pathway, ligand induced activation results in the activation of NF-κB-inducing kinase (NIK), which phosphorylates the IKK

complex that contains two IKKα subunits (but not NEMO) (Senftleben *et al.*, 2001). The mechanism of NIK regulation is tightly controlled: the basal level of NIK is kept low by a TRAF-cIAP destruction complex and signal-induced non-canonical NF-κB signalling involves NIK stabilisation (Sun, 2011). Activated IKKα then phosphorylates p100 and leads to the processing and liberation of the p52/RelB active heterodimer (Gilmore, 2006; Liu and Malik, 2006). Genetic evidence suggests that this NF-κB pathway regulates important biological functions, such as lymphoid organogenesis, B-cell survival and maturation, dendritic cell activation, and bone metabolism (Dejardin, 2006). Moreover, deregulated non-canonical NF-κB signalling is associated with lymphoid malignancies (Sun, 2011).

NF-κB activity is also controlled by additional regulatory mechanisms. These include regulation of nuclear import and export of NF-κB dimers, regulation of the recruitment of NF-κB dimer to its target genes and regulation of NF-κB-mediated transcriptional activity. These are primarily mediated by two mechanisms, post-translational modification of NF-κB proteins and synergistic (or antagonistic) interactions between NF-κB and other transcriptional proteins, as well as transcriptional coactivators or corepressors (Chen and Greene, 2004; Perkins, 2006; Huang *et al.*, 2010). Nevertheless, NF-κB protein phosphorylation has emerged as an important mechanism regulating NF-κB activity and NF-κB-mediated gene transcription (Chen and Greene, 2004). Stimulus-dependent phosphorylation of p65 was the first regulatory event that was recognised to occur downstream from IκB degradation (Naumann and Scheidereit, 1994; Neumann *et al.*, 1995). Recent

studies have also implicated the transcriptional activity of NF-κB to be controlled by phosphorylation of p65 at multiple serine residues (Geisler *et al.*, 2007; Reber *et al.*, 2009; Huang *et al.*, 2010; Moreno *et al.*, 2010).

2.6   Crosstalk between NF-κB and PPARα

The onset of inflammatory gene expression is driven by the transcription factor NF-κB, whose transcriptional activity is regulated at multiple levels (Vanden Berghe *et al.*, 2003). PPARα is a prototype of metabolic nuclear receptor and has been shown to orchestrate physiological responses to inflammatory stimuli (Delerive *et al.*, 1999a; Lefebvre *et al.*, 2006). It has been reported that PPARα inhibits expression of IL-6, prostaglandin, and COX-2 via repression of NF-κB signalling in aortic smooth muscle cells, and thus possibly reducing the risk for atherosclerosis (Staels *et al.*, 1998b). Several studies have delineated the cellular and molecular mechanisms of the anti-inflammatory action of PPARα, which negatively interferes with the inflammatory response by antagonising the NF-κB signalling pathway (Figure 2.6).

Transcriptional activity of NF-κB is mediated by interaction with cofactors that remodel chromatin and bridge the DNA-bound transcription factors to the basal transcription machinery. The p300/CBP proteins appear to be the basal components of functional NF-κB transcription complex (Gerritsen *et al.*, 1997). Therefore, a decrease in cofactor availability would strongly affect transcription. Feige and co-workers (2006) have demonstrated that binding of ligands to PPARα modifies the conformation of the PPARα LBD
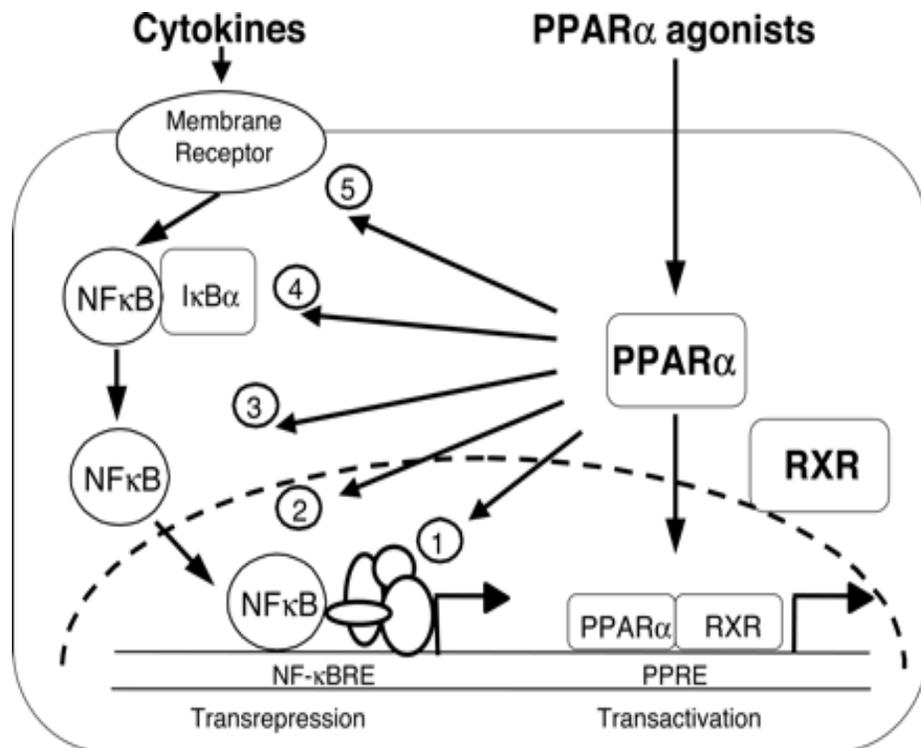
Figure 2.6: Interaction of PPARα at several levels of the NF-*κ*B signalling pathway.
PPARα interference with the NF-*κ*B pathway through (1) interference with activation of the transcription initiation complex via cofactor interaction, (2) lowering of transcription factor expression levels, (3) inhibition of nuclear translocation of inflammatory transcription factors, (4) direct protein-protein interaction and induction of inhibitory protein expression such as I*κ*Bα, and (5) down-regulation of the expression of signal transducing receptor components (Adapted from Zambon *et al.*, 2006).

and unmasking the interaction area for coactivators such as SRC1 and p300/CBP. These coactivators are equipped with histone acetyl transferase (HAT) activity, which causes chromation decondensation and target gene activation. Hence, the anti-inflammatory mode of action of activated PPARα may impair the recruitment of transcriptional cofactors to the NF-κB-DNA complex.

Furthermore, transcriptional activity of transcription factors also depends on their level of expression. PPARα activators were shown to decrease expression levels of p50 and C/EBPβ in the livers of wild-type mice, but not in PPARα-deficient mice (Kleemann *et al.*, 2003). Subsequently, cytokine-activated signal transduction pathways can also modulate the transition of inactive to active forms of transcription factors via altering their phosphorylation. It has been reported that inhibition of both IKK activity and TNF-α-induced IκBα phosphorylation by PPARα agonist, fenofibrate in human umbilical vein of endothelial cells, leading to enhanced cytosolic IκBα stability and the promotion of NF-κB cytoplasmic sequestering through its tight association with IκBα (Okayasu *et al.*, 2008). Moreover, a synthetic glucocorticoid, dexamethasone was shown to inhibit p65 phosphorylation at Ser536 in wild-type, but not in PPARα knockout mice (Cuzzocrea *et al.*, 2008). Hence, it is noteworthy that modulation of NF-κB phosphorylation status constitutes another level of control of PPARα.

A direct interaction of PPARα with AP-1 and NF-κB has also been characterised by Staels *et al.* (1998a) and Delerive *et al.* (1999a). PPARα

inhibits vascular inflammation in aortic smooth muscle cells by physical interactions with c-Jun and p65. Glutathione-S-transferase (GST) pull-down assays revealed that PPARα physically interacts with p65 via its Rel homology domain which mediates homo- and hetero-dimerisation and interaction with IκB (Delerive *et al.*, 1999a). On the other hand, fibrates (a synthetic PPARα ligand) can induce the IκBα mRNA and protein expression in both smooth muscle cells and hepatocytes, resulting in sequestration of NF-κB in the cytoplasm and reduction in its DNA-binding ability. This induction takes place in the absence of PPRE, but requires the presence of NF-κB and Sp1 elements in the IκBα promoter sequence as well as DRIP250 cofactor (Vanden Berghe *et al.*, 2003). These findings are corroborated by the results from PPARα-null mice, which showed the induction of IκBα expression is PPARα-dependent (Delerive *et al.*, 2000).

In fact, a bidirectional antagonism between the PPARα and NF-κB signalling pathways exists (Delerive *et al.*, 1999a). Several lines of evidence have revealed a reciprocal effect of NF-κB on PPARα transcript levels and transcriptional interference between PPARα and NF-κB. For example, suppression of human and mouse PPARα promoter activity by IL-1β was recapitulated by overexpression of NF-κB subunit p50 and p65, and was abolished upon deletion of putative NF-κB binding sites (Stienstra *et al.*, 2010). This indicates that NF-κB interferes with the ability of PPARα to activate gene transcription. Research by Cabrero *et al.* (2002) also showed that the increased NF-κB activity led to down-regulation of fatty acid oxidation metabolism mediated by PPARα and the subsequent intracellular lipid accumulation in

skeletal muscle cells. Furthermore, previous studies also suggested that age-associated reductions in PPARα mRNA levels are mediated through enhanced NF-κB activation (Poynter and Daynes, 1998) and co-transfection of increasing amounts of p65 led to a dose-dependent inhibition of a PPRE-driven promoter construct (Delerive *et al.*, 1999a). On the other hand, Wunderlich *et al.* (2008) revealed that inhibition of the NF-κB signalling pathway by inactivating the NF-κB essential modulator (NEMO) gene in rodent liver would lead to a decrease in the expression of PPARα.

CHAPTER 3

MATERIALS AND METHODS

3.1 General preparation

All glassware and plasticware (pipette tips, microcentrifuge tubes, centrifuge tubes, etc.) were autoclaved at 121°C for 20 min and at the pressure of 975 kPa prior to use. All solutions used in handling DNA and RNA work were also autoclaved using the above conditions; phosphate buffered saline (PBS), however, was autoclaved at 121°C for 15 min and at the pressure of 975 kPa. The consumables used for this study and their suppliers are listed in Table 3.1.

3.2 Cell culture techniques

3.2.1 Maintenance of cells in culture

HepG2 is human hepatocellular carcinoma cell line. It is a kind gift from Associate Professor Dr. Tengku Sifzizul Tengku Muhammad, University Malaysia Terengganu. HepG2 cell was maintained in filter-sterilised Eagle's minimum essential medium (MEM) supplemented with 2.2 g/L sodium bicarbonate, 10 μM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μM non-essential amino acid, and 10% (v/v) heat-inactivated (30 min, 56°C), filter-sterilised foetal bovine serum (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 flask (25 cm^2) was replaced every 3 days.

Table 3.1   Materials used in the project and their suppliers.

Materials	Suppliers
Bromophenol blue, Ethylenediaminetetraacetic acid (EDTA), Isopropanol, Acetone, Ammonium persulphate (APS), Acrylamide-bisacrylamide^{TM} 37.5:1 (30:0.8) 40% (w/v) solution, Phosphate-buffered saline (PBS), Tween$^{®}$ 20, Actinomycin D, Guanidine hydrochloride	Amresco
QuantiFast^{TM} SYBR$^{®}$ Green RT-PCR Kit	Qiagen
Ethanol	HmbG Chemicals
Sodium chloride (NaCl), Bovine serum albumin (BSA)-fraction V, Methanol, 2-mercaptoethanol	Merck
Penicillin/Streptomycin, Trypsin-EDTA, 1-bromo-3-chloropropane (BCP)	Sigma Aldrich
Minimum essential medium (MEM), Foetal bovine serum (FBS), Sodium pyruvate, Non-essential amino acids	Gibco
Prestained protein marker (broad range)	Biolabs
HepG2 cell line	American Type Culture Collection
TRI-Reagent$^{®}$ LS	Molecular Research Centre
Sodium dodecyl sulfate (SDS), Glycerol	Fisher Scientific
Tris, Glycine	Promega
NE-PER$^{®}$ Nuclear and Cytoplasmic Extraction Reagents	Pierce
Reverse primer, Forward primer	Research Biolabs
Protease inhibitor cocktail Set I, IKK inhibitor II (Wedelolactone), NF-κB activation inhibitor IV, IKK-2 inhibitor (SC-514), Tumor necrosis factor-alpha (TNF-α)	Calbiochem
Cell scrapers, Tissue culture flasks (25 cm^2 and 75 cm^2), Cryo-vials	Techno Plastic Products (TPP)

The cell culture was washed twice with 2 ml of PBS before complete growth medium with 10% (v/v) FBS were added into the tissue culture flask.

3.2.2   Subculturing of cells

Subculturing of cells was performed after the cells achieved 90% confluence. Initial medium in the tissue culture flask was discarded and the cells were washed twice with phosphate buffered saline (PBS) to remove all trace of serum which contained trypsin inhibitor. Subsequently, 1 ml of 0.25% (w/v) trypsin was added to the cells and the culture flask was incubated at 37°C, 5% (v/v) CO_2 incubator for 10 – 15 min to disperse the cells from the growth surface on the flask base. After incubation, the cells were inspected under inverted microscope in order to confirm that all cells were detached. Then, 2 ml of complete growth medium was added to achieve a final volume of 3 ml in the tissue culture flask. The medium was gently transferred into a sterile 15 ml centrifuge tube and centrifuged at 500 g for 5 min.

Following centrifugation, the pellet was resuspended gently in 2 ml of complete growth medium and the volume of cells were then divided evenly into new 25 cm^2 tissue culture flasks. Complete growth medium and 10% (v/v) of FBS were then added into each of the flask to a final volume of 3 ml. The cells were incubated in a humid incubator (37°C, 5% (v/v) CO_2).

3.2.3   Cells storage

When the cells in 25 cm^2 tissue culture flask reached around 90% confluency, they were trypsinised as described in Section 3.2.2. They were centrifuged at 500 g for 5 min. The supernatant was removed and the cells were resuspended in 1 ml of FBS supplemented with 10% (v/v) dimethyl sulphoxide (DMSO). The cell suspension was dispensed into sterile cryo-vial and subsequently stored at -80°C overnight before it was transferred to the liquid nitrogen tank.

3.2.4   Thawing of frozen cells

A cryo-vial was removed from the liquid nitrogen tank and placed in a 37°C waterbath for approximately 2 min. The vial was removed as soon as the contents were thawed. The content was transferred to a 15 ml centrifuge tube containing 9 ml of MEM and centrifuged at 500 g for 5 min. The supernatant was discarded and the cells were resuspended in an appropriate volume of complete medium and plated out in tissue culture flask. On the following day, the cells were washed twice with PBS to remove any leftover DMSO and fresh medium with FBS were added.

3.3   Treatment of HepG2 cells with cytokine, TNF-α

HepG2 cells were seeded into 25 cm^2 tissue culture flasks and allowed to grow until the point of ~70% confluence. Prior to incubation with mediators, the cells were washed twice with PBS and pre-incubated for 2 h in medium containing reduced 0.5% (v/v) heat-inactivated FBS. The medium

was then removed, washed twice with PBS before treatment. Dose response of TNF-α treatment was carried out by addition of different concentrations of TNF-α at 10 ng/ml, 20 ng/ml, 50 ng/ml and 100 ng/ml in fresh medium supplemented with 0.5% (v/v) heat-inactivated FBS for 24 h. Whereas, the investigation of time-course dependency effect of TNF-α was determined over a 24 h period of incubation with a single dose of TNF-α. The time intervals used were 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h. For the control samples, cells were left untreated without any mediator. The cells were incubated at 37°C in a humid atmosphere of air containing 5% (v/v) CO_2.

3.4   Treatment of cells with actinomycin D (mRNA stability test)

The mRNA stability can be rapidly modulated to alter the expression of specific genes, thereby providing flexibility in affecting changes in patterns of protein synthesis. In order to determine whether the cytokine-regulatory effect on the level of PPARα mRNA expression was caused at the level of PPARα mRNA stability, HepG2 cells were stimulated with or without an appropriate amount of TNF-α for 24 h. Following this, 5 μg/ml of actinomycin D was added to the cells for 2 h or 4 h, respectively. For the control sample, the addition of actinomycin D was excluded and the cells were further incubated at 37°C for 2 h or 4 h. Subsequently, total RNA was extracted as stated in Section 3.6.

3.5 Pre-treatment of cells with inhibitors

Prior to any treatment, the HepG2 cells were pre-incubated in medium containing reduced amount of FBS as described in Section 3.3. The medium was then removed and washed twice with PBS. In order to investigate the specific cell signalling pathway involved in the regulation of NF-κB on PPARα expression, different dosages of Wedelolactone (10 μM, 25 μM, and 50 μM) were used and the concentration of IKK-2 inhibitor (SC-514) and NF-κB activation inhibitor IV used were 50 μM and 200 nM, respectively. After 2 h of incubation, TNF-α was added into the flasks and incubated for an appropriate period of time. For the determination of time-course dependency effect of Wedelolactone, a single dosage was added into the flasks and incubated for 30 min, 1 h, 2 h, and 3 h prior to TNF-α treatment. Since Wedelolactone was dissolved in DMSO, 0.01% (v/v) DMSO were added with 0.5% (v/v) heat-inactivated FBS and TNF-α to the culture medium and used as control. The cells were then incubated at 37°C in a humid atmosphere of air containing 5% (v/v) CO_2.

3.6 Isolation of total cellular RNA

HepG2 cells were cultured and maintained in a 25 cm^2 tissue culture flask until they reached 70% confluence. Total cellular RNA was isolated from cells cultured in 25 cm^2 tissue culture flasks using the TRI-Reagent LS (Molecular Research Center) according to the manufacturer's instruction.

Cells were rinsed twice with PBS and then homogenised with 0.75 ml of TRI Reagent LS in the flask and were scraped by using cell scraper

(TPP). The homogenate was then transferred to a 1.5 ml microcentrifuge tube and incubated at room temperature for 5 min to allow complete dissociation of nucleoprotein complex. Subsequently, the homogenate was added with 0.1 ml of BCP and shaken vigorously for 15 sec. The mixture was left at room temperature for 15 min and centrifuged at 12,000 g for 15 min at 4°C. Following centrifugation, the mixture was separated into a lower pink phenol-chloroform phase, interphase and the colourless upper aqueous phase which contained the protein, DNA and RNA, respectively. The aqueous phase was transferred to a new microcentrifuge tube and RNA was precipitated by the addition of 0.5 ml of isopropanol. The mixture was incubated at room temperature for 10 min and then centrifuged at 12,000 g for 8 min at 4°C. Subsequently, the RNA pellet was washed with 1 ml of 75% (v/v) ethanol and centrifuged at 7,500 g for 5 min at 4°C. The RNA pellet was air-dried for 15 – 20 min, resuspended in 30 µl of ddH$_2$O and incubated at 55°C for 15 min before it was stored at -80°C.

The concentration and purity of isolated RNA were determined by measuring the absorbance values at 230 nm, 260 nm and 280 nm using the NanoDrop^{TM} 1000 Spectrophotometer (Thermo Scientific). The quality of RNA was assessed by subjecting a small aliquot to electrophoresis on 1% (w/v) agarose-formaldehyde gel, in order to check the integrity of total RNA extracted.

3.7 Real-Time RT-PCR

Real-Time PCR was carried out using QuantiFast^{TM} SYBR$^{®}$ Green RT-PCR Kit (Qiagen) according to the manufacturer's instructions. All reactions were assembled on ice and performed in a final volume of 25 µl in reactions containing 12.5 µl of 2× QuantiFast SYBR Green I, 5.25 µl of RNase-free water, 2.5 µl of each forward and reverse primer (10 µM) (Table 3.2), 0.25 µl of QuantiFast RT Mix and 2 µl of 50 ng/ml of RNA. The samples were then placed in MyiQ^{TM} Real-Time PCR Detection System (Bio-Rad) for quantitative PCR. The protocols are shown in Table 3.3 following Chew *et al.* (2007). A melt curve analysis was carried out at the end of the quantitative PCR cycle. The quantity of mRNA target was normalised against a chosen housekeeping gene, β-actin, which acted as internal control, in order to determine the relative mRNA expression of the target gene.

3.8 Protein-associated techniques

3.8.1 Isolation of total proteins

Total protein was extracted by using TRI-reagent LS. After the aqueous phase (RNA) overlying the interphase (DNA) described in Section 3.6 was removed, 0.3 ml of 100% ethanol was added for initial homogenisation. The sample was mixed by inversion and was allowed to stand for 3 min. Finally, DNA was sedimented by centrifugation at 2,000 g for 5 min at 4ºC.

After precipitation of DNA, 0.4 ml of phenol-ethanol supernatant was aliquoted into a 1.5 ml microcentrifuge tube. The proteins were

Table 3.2   Nucleotide sequences of the primers used for Real-Time RT-PCR.

Primer	Sequence (5' – 3')	Expected size/bp	References
β-actin_F	TCACCCTGAAGTACCCCATC	489	Chew *et al.*, 2006
β-actin_R	CCATCTCTTGCTCGAAGTCC		
PPARα_F	CCGTTATCTGAAGAGTTCCTG	440	Chew *et al.*, 2006
PPARα_R	GTTGTGTGACATCCCGACAG		
ApoAI_F	CTGGCCACTGTGTACGTGGATG	310	Matsuura *et al.*, 2007
ApoAI_R	TGGCGGTAGAGCTCCATCTCCT		
ApoB_F	CTGGGAAAACTCCCACAGCAAG	414	Matsuura *et al.*, 2007
ApoB_R	CCACATTTTGAATCCAGGATGCAG		

Table 3.3   Real-Time RT-PCR cycle and melting curve cycle (Chew *et al.*, 2007).

Step	Temperature	Time	Cycle
Reverse transcription	50°C	10 min	1
PCR initial activation step	95°C	5 min	1
Denaturation	95°C	10 sec	
Annealing	60°C	30 sec	35
Extension	72°C	30 sec	
Melting curve	95°C	1 min	1
	55°C	1 min	1
	60°C – 95°C (increase set point temperature by 0.5°C)	10 sec	71

precipitated by adding 3 volume of acetone and mixed by inversion for 15 sec. The sample was stored for 10 min at room temperature. Next, the protein was sedimented at 12,000 g for 10 min at 4ºC. The phenol-ethanol supernatant was discarded and the protein pellet was dispersed in 0.5 ml of G:E:G wash solution (0.3 M guanidine hydrocholoride in 95% (v/v) ethanol, 2.5% (v/v) glycerol) by using the pipette tip. After dispersing the pellet, another 0.5 ml of the G:E:G wash solution was added to the sample and stored for 10 min at room temperature. The protein was sedimented at 8,000 g for 5 min. Following centrifugation, the wash solution was removed and two more washes were performed in 1 ml each of the G:E:G wash solution. The pellet was dispersed by vortexing after each wash to efficiently remove residual phenol. The final wash was performed in 1 ml of ethanol containing 2.5% (v/v) glycerol for 10 min and protein was sedimented at 8,000 g for 5 min. After the alcohol was discarded, the tube was inverted and the pellet was air-dried for 10 min at room temperature. Finally, 0.2 ml of 1% of SDS solvent was added to the protein pellet and dispersed gently for 20 min to solubilise the pellet. The solubilised proteins were used immediately for western blotting (Section 3.8.4) or stored at -20ºC until further downstream processing.

3.8.2   Isolation of nuclear and cytoplasmic proteins

Nuclear and cytoplasmic proteins were isolated from cells cultured in 25 cm^2 tissue culture flasks using NE-PER$^®$ Nuclear and Cytoplasmic Extraction Reagents (Pierce). The procedures were carried out according to the manufacturer's instructions and the Protease Inhibitor Cocktail Set I

(Calbiochem) was added to reagents CER I and NER from the concentrated stock.

Firstly, cells were rinsed twice with PBS and were scraped using cell scraper after adding 1 ml of PBS into the flask. The packed cell volume was then transferred to a 1.5 ml microcentrifuge tube and centrifuged at 500 g for 3 min at 4°C. The supernatant was discarded and the cell pellet was allowed to dry. Subsequently, 200 μl of ice-cold CER I was added to the cell pellet and vortexed vigorously for 15 sec. The mixture was incubated on ice for 10 min before 11 μl of ice-cold CER II was added. It was vortexed vigorously for 5 sec, incubated on ice for 1 min, and then vortexed vigorously for another 5 sec. After centrifugation at 16,000 g for 5 min at 4°C, the supernatant fraction (which was the cytoplasmic extract) was transferred immediately to a pre-chilled 1.5 ml microcentrifuge tube and stored at -80°C.

Next, the insoluble fraction which contained nuclei was resuspended in 100 μl of ice-cold NER and vortexed vigorously for 15 sec. The sample was incubated on ice and vortexed for another 15 sec every 10 min, for a total of 40 min. Finally, the sample was centrifuged at 16,000 g for 10 min at 4°C. The supernatant fraction which was the nuclear extract was transferred immediately to a pre-chilled 1.5 ml microcentrifuge tube and stored at -80°C.

3.8.3   Bio-Rad D_c protein assay

The concentration of extracted proteins (Section 3.8.1 and Section 3.8.2) was determined by using the Bio-Rad D_c Protein Assay reagent

kit (Bio-Rad) according to the manufacturer's instructions. For the preparation of working reagent A', 20 µl of reagent S was added to each millitre of reagent A that was needed for the analysis. Next, a standard curve was prepared for each assay using suitable dilution of 1.52 mg/ml BSA solution in order to produce concentrations of 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml, 1.2 mg/ml and 1.4 mg/ml. After 5 µl of samples or standards were pipetted into a well of a 96-well micro-titer plate, 25 µl of reagent A' was added into each well followed by the addition of 200 µl of reagent B. It was mixed for 15 sec and then incubated for 15 min. Subsequently, absorbances were read at 750 nm using the ELISA plate reader, infinite M200 (Tecan®). The protein concentration of the experimental samples was calculated from a standard curve.

3.8.4   Western blot

3.8.4.1   Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed under reducing conditions following the method by Laemmli (1970). Electrophoresis was carried out using the Mini-PROTEAN® Tetra Cell (Bio-Rad) with the gel apparatus being assembled as described by the manufacturer. In this system, two sequential gels were used: resolving or separating gel and stacking gel that containing 10% (w/v) and 5% (w/v) acrylamide respectively. These gels were prepared from stock solutions described in the Table 3.4.

Table 3.4   Composition of stacking and separating gels used for SDS-PAGE. Volume shown here are for preparation of two gels.

Gel component	10% (w/v) Resolving gel	5% (w/v) Stacking gel
SDS-PAGE upper gel buffer	-	1.25 ml
SDS-PAGE lower gel buffer	2.5 ml	-
40% (w/v) acrylamide:bisacrylamide (37.5:1)	2.5 ml	0.625 ml
ddH$_2$O	4.89 ml	3.07 ml
10% (w/v) APS	100 μl	50 μl
TEMED	10 μl	5 μl

After assembling the gel cassette, a comb was placed into it and the glass plate was marked 1 cm below the comb teeth. The resolving gel monomer solution was poured to the mark. Butanol was layered on top of the monomer solution to exclude any air bubbles and the gel was allowed to polymerise for 40 min. Once the gel had solidified, the gel surface was rinsed completely with ddH_2O and the upper surface was dried with filter paper. Next, the stacking gel monomer solution was poured on top and the desired comb was inserted. After the stacking gel polymerised for 40 min, the comb was removed and the wells were rinsed with ddH_2O. The gels were then placed in the electrophoresis tank and the assembly (upper chamber) and the tank (lower chamber) were filled with SDS-PAGE running buffer (Table 3.5). Before the protein samples were loaded onto the gel, they were mixed with 5 μl of loading buffer and heated at 100°C for 2 min. For the first lane of each gel, 8 μl of prestained protein marker (Biolabs) were loaded. Electrophoresis was carried out at 100 V in a cool chamber at 4ºC.

3.8.4.2   Protein electrophoretic transfer

The protein electrophoretic transfer was carried out using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions. The protein was transferred to Immobilon®-P transfer membrane (Millipore). Briefly, the gel was removed from the glass plates and the stacking gel was removed. The gel was equilibrated in pre-chilled Towbin transfer buffer (Table 3.5) for 15 min, while the membrane, filter paper (both cut to the dimensions of the gel), and fiber pads were also pre-soaked in the transfer buffer. Next, the membrane was placed on the gel

Table 3.5   Solutions for western blot

Solution	Composition
SDS-PAGE upper gel buffer	0.5 M Tris, 10% (w/v) SDS, pH 6.8
SDS-PAGE lower gel buffer	1.5 M Tris, 10% (w/v) SDS, pH 6.8
SDS-PAGE running buffer	25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS
Towbin transfer buffer	25 mM Tris, 192 mM Glycine, 20% (v/v) methanol
SDS-PAGE gel loading buffer	0.12 M Tris, 25% (v/v) glycerol, 2% (w/v) SDS, 0.004% (w/v) bromophenol blue, 5% (v/v) 2-mercaptoethanol, pH6.8
Tris-buffered saline-Tween 20 (TBS-T) (washing solution)	10 mM Tris, 200 mM NaCl, 0.1% (v/v) Tween 20, pH 7.4
Blocking solution	1% (w/v) BSA, 10 mM Tris, 200 mM NaCl, 0.1% (v/v) Tween 20
Stripping solution	0.4 M Glycine, 0.2% (w/v) SDS, 2% (v/v) Tween 20, pH 2.2

and sandwiched between the filter papers and fiber pads. Air bubbles, if any, were removed by using a glass tube to roll out the air bubbles. It was then placed in the blotting cassette and subjected to electrophoresis at 4°C at a constant voltage of 100 V for 1 h 15 min in a tank containing transfer buffer. The transferred protein in the membrane was then immunodetected as described in Section 3.8.4.3. If not used immediately, the membrane was kept between filter papers at -80°C until further processing.

3.8.4.3 Immunodetection of proteins

The detection of immunoreactive proteins on western blot was performed by using the SNAP i.d. Protein Detection System (Millipore) according to the manufacturer's instructions. The dry membrane was re-wet with 100% methanol and then rinsed with ddH$_2$O. After the system was connected to a vacuum source, the blot was assembled. Briefly, the inner white face of the triple well blot holder was wet with ddH$_2$O before the pre-wet membrane was placed with the protein side down. Then, the spacer was placed on top of the blot and blot roller was used to remove any air bubbles.

Membrane was probed immunochemically as described below. Non-specific protein binding sites on the membrane were blocked by incubating the membrane in blocking solution (Table 3.5) for 5 min. After the blocking solution was removed by turning the vacuum on, the membrane was incubated with 1 ml of blocking solution containing 1:1000 dilution of the primary antibody (Table 3.6) for 10 min. The membrane was then washed three times with 10 ml of washing solution (Table 3.5) before the membrane was

Table 3.6   Antibodies used in the project and the suppliers

Antibodies	Suppliers
#4967 β-actin	Cell Signaling
#2682 IKKα	Technology®
#2681 Phospho-IKKα (Ser180)/IKKβ (Ser181)	
#9242 IκBα	
#2859 Phospho-IκBα (Ser32)	
#9248 IκBβ	
#4921 Phospho-IκBβ (Thr19/Ser23)	
#3035 NF-κB p105/p50	
#4806 Phospho-NF-κB p105 (Ser 933)	
#4882 NF-κB2 p100/p52	
#4810 Phospho-NF-κB2 p100 (Ser 866/870)	
#3034 NF-κB p65	
#3037 Phospho-NF-κB p65 (Ser276)	
#3039 Phospho-NF-κB p65 (Ser468)	
#3031 Phospho-NF-κB p65 (Ser536)	
#7074 Anti-rabbit IgG, HRP-linked	
#2032S LaminA/C	
#E3107 PPARα	Santa Cruz Biotechnology

incubated in 1 ml of blocking solution containing of the secondary antibody, horseradish peroxisdase (HRP)-conjugated anti-rabbit IgG (1:3000 dilution) for 10 min. Lastly, another set of washes were performed.

3.8.4.4   Chemiluminescent detection

The Immobilon Western Chemiluminescent HRP substrate (Millipore) was used to detect the membrane bound antigen-antibody complexes. The protocol was modified from the manufacturer's instructions and optimised for the detection for 3×8.5 cm membrane. An equal volume of luminal reagent and peroxide solution were mixed. Subsequently, after the working HRP substrate was added onto the membrane's surface and incubated approximately 5 – 10 min, the excess detection reagent was drained off. Next, the membrane was placed protein side-up and a piece of clean and transparent plastic wrap was placed onto it. A small sheet of Fuji Medical X-ray film was placed on top of the membrane for an appropriate duration. Re-exposure for a further period of time was dependent upon the intensity of the signal obtained. X-ray films were developed using developer and fixer solutions (Fuji). Pre-stained protein markers present on the membrane were then aligned with the developed image in order to verify the expected protein size and the immunodetected protein bands were analysed using the AlphaView® software (Alpha Innotech) for FluorChem systems.

3.8.4.5 Membrane stripping

The membrane bound antigen-antibody complexes were stripped by using the stripping solution (Table 3.5) at 240 min^{-1} on the orbital shaker, OS 5 Basic (IKA Yellowline) for 10 min. The membrane was then washed twice for 15 min for each wash with washing solution and soaked in 100% methanol for a few seconds. The membrane was air-dried, sandwiched between the filter papers and stored at -80°C for the next immunoblotting.

3.9   Statistical analysis

Quantitative data were expressed as means ± standard error of mean (SEM). Statistical significance was determined by using one-way analysis of variation (ANOVA) with the post hoc test, which is the least significant difference (LSD) method available in Statistical Package for the Social Sciences (SPSS) version 15.0 software. $P<0.05$ was considered to be significant, $P<0.01$ was considered to be highly significant, and $P<0.001$ was considered to be extremely significant.

CHAPTER 4

RESULTS

4.1   Assessment of total cellular RNA purity and integrity

The cells were grown until they reached 60% – 70% confluency and the total cellular RNA was isolated from HepG2 cells using the TRI-Reagent LS (Molecular Research Center) as described in Section 3.6. Data analyse of the concentration and purity of the RNA samples were measured by the readings derived from 230 nm, 260 nm and 280 nm using NanoDrop 1000 Spectrophotometer (Thermo Scientific). This is because nucleic acids and proteins have an absorption maximum at 260 nm and 280 nm, respectively. Absorption at 230 nm can be caused by contamination by phenolate ion, thiocyanates, and other organic compounds. The A_{260}/A_{280} ratio is used to assess the purity of RNA. For the A_{260}/A_{230} ratio, it is a secondary measure of RNA purity and often higher than the respective A_{260}/A_{280} ratio if a "pure" RNA was obtained. In this study, all the isolated RNA samples had an A_{260}/A_{280} ratio of ~2.0 and A_{260}/A_{230} ratio of 1.9 – 2.2 (Appendix A). These values are generally accepted as "pure" value for RNA (Auburn, 2004). However, the sample that obtained a lower A_{260}/A_{230} ratio than its respective A_{260}/A_{280} ratio indicated that there were minor impurities found in the RNA samples. This may due to the carried over of TRI Reagent LS, which contains the combination of phenol and guanidine thiocyanate that could cause higher reading in A_{230}.

The integrity of the total cellular RNA extracted was assessed by an electrophoresis process in 1% (w/v) denaturing agarose-formaldehyde gel (Section 3.6) and viewing the total RNA under UV transilluminator. The two distinct bands on the agarose-formaldehyde gel observed in the gel indicated intact 28S and 18S rRNA bands. As shown in Figure 4.1, the 28S rRNA band was approximately twice the intensity of the 18S rRNA band. This indicates that the total RNA extracted was of high purity and integrity. The 2:1 ratio (28S:18S) is a good indication of intact RNA (Chacko, 2005).

4.2   Real-Time RT-PCR

Real-Time RT-PCR were employed in the studies by using QuantiFast^{TM} SYBR$^{®}$ Green RT-PCR Kit (Qiagen) as described in Section 3.7 to examine the mRNA expression profile of PPARα in HepG2 cells in response to TNF-α. It is one of the most sensitive and reliable quantitative methods for gene expression analysis as a result of physiology, development, or pathophysiology (Yuan *et al.*, 2006). This system conveniently comes with software which allows automated calculation of the relative gene expression called Gene Expression Analysis for MyiQ Real-Time PCR Detection System. Real-Time RT-PCR amplifies a specific target sequence in the sample, in which its amplification progress is monitored using SYBR Green I as fluorophore. For this study, relative quantification was used. The comparison between expressions of target gene versus a reference gene was firstly generated before the relative expressions of experimental samples were compared with reference samples (control) (Pfaffl, 2001). The housekeeping gene, β-actin, was chosen as endogenous control for each normalisation.

28S rRNA

18S rRNA
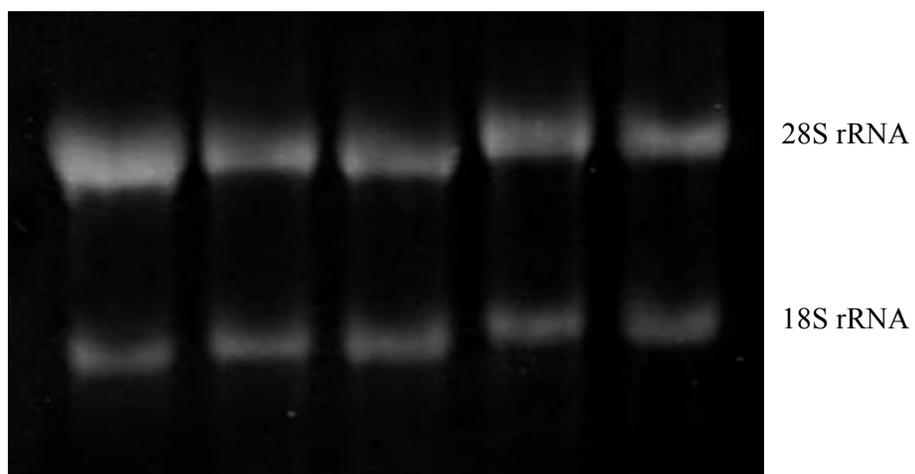
Figure 4.1: Agarose-formaldehyde gel electrophoresis of total cellular RNA isolated from HepG2 cells using TRI-Reagent LS (Molecular Research Center).
A small aliquot of RNA was subjected to 1% (w/v) agarose-formaldehyde gel electrophoresis. The presence of the two 28S and 18S rRNA bands in 2:1 ratio shows the isolated RNA was degradation free.

In order to ensure the reliability, at least three independent experiments were carried out and analysis was performed in triplicate for each experiment. A representation of the sigmoidal-shaped amplification plots is shown in Figure 4.2. It shows that there was a significant fold increase in the PCR amplification for both PPARα (target gene) and β-actin (housekeeping gene). The point whereby the cycle number crosses the arbitrary line (or the threshold) is called the C_T (cycle threshold) value. It is auto-calculated by the software and then translated into relative expression folds by using Pfaffl formula (Appendix B) (Pfaffl, 2001). In addition, C_T value is inversely proportional to the amount of target nucleic acid in the sample. Thus, the β-actin which has lower C_T value mean (20.43) than the PPARα C_T value mean (23.97) showed that it is greater expressed than PPARα in HepG2 cells, as expected.

For SYBR Green based amplicon detection, it is important to run a dissociation curve following the Real-Time RT-PCR (Figure 4.3). This is due to the fact that SYBR Green will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from any misannealled primer. The presence of a single, sharp peak in the respective β-actin and PPARα melt curve dissociated chart indicated that a specific desired product was amplified with the primers set (Table 3.2). The melting temperature (T_m) for β-actin and PPARα were found to be 88.51°C and 87.00°C, respectively.
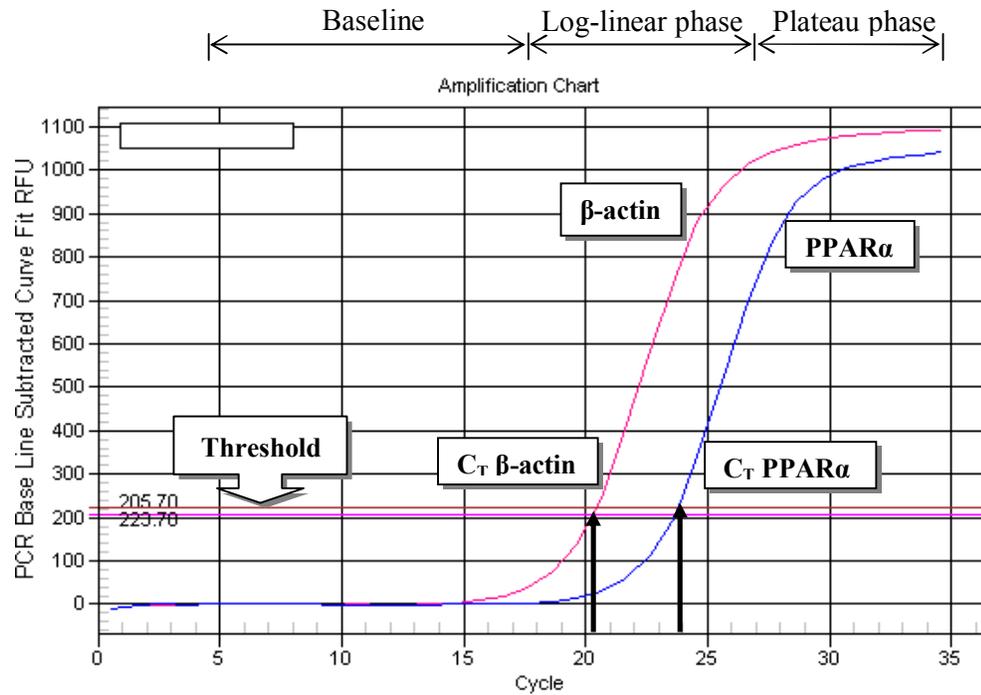
Figure 4.2: Real-Time RT-PCR amplification plot for PPARα and β-actin genes.
The graphical representative illustrating the increased in arbitrary fluorescence units (which are fold increase over background fluorescence) (Y-axis) with each cycle number (X-axis). Three phases could be observed for PCRs: baseline phase (background signal or lag phase), log-linear phase (exponential amplification phase), and plateau phase (end-point phase).

(a)

(b)
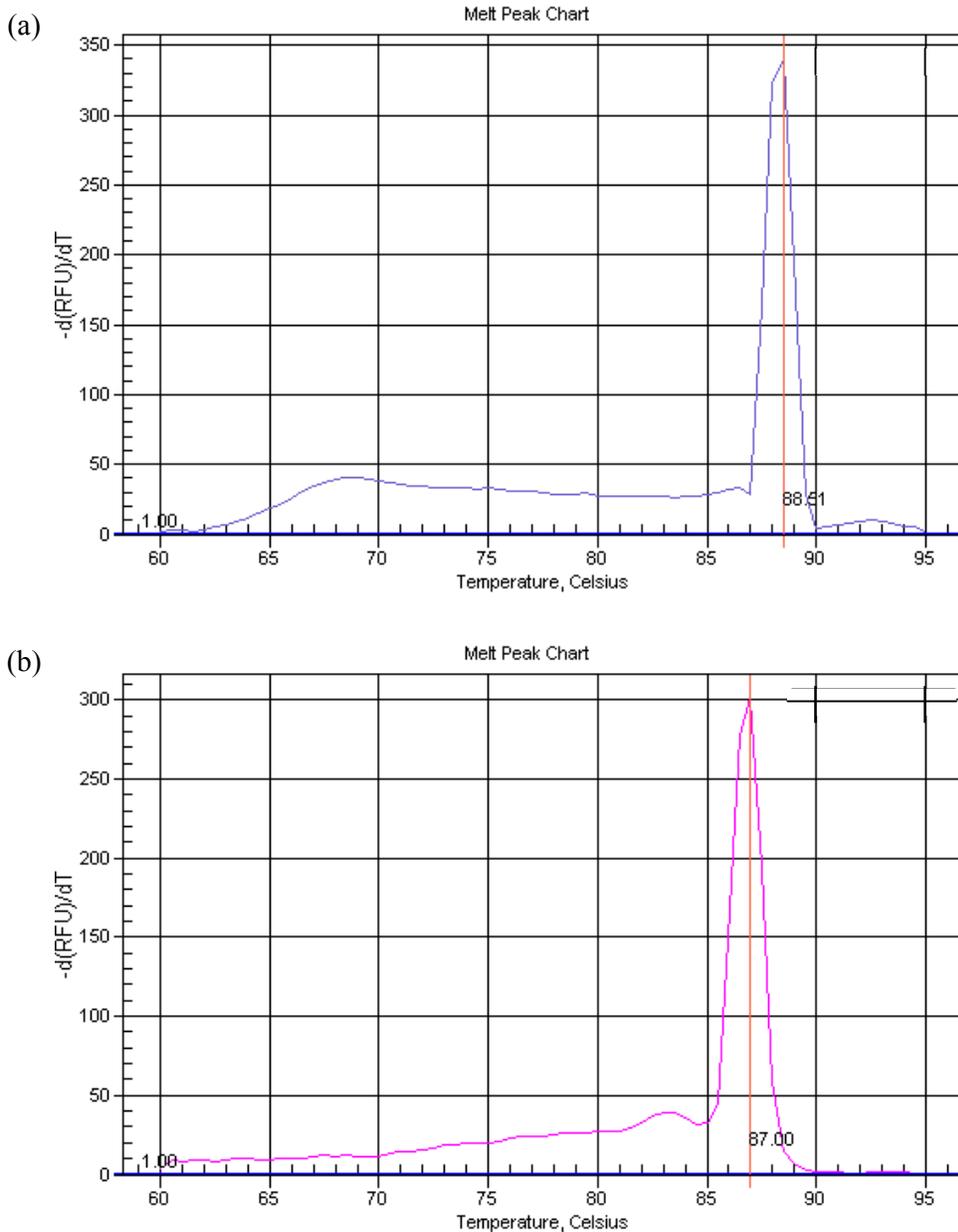
Figure 4.3: Graphical representation of a melting peak in Real-Time RT-PCR.
Melting curve analysis of (a) β-actin and (b) PPARα was performed. The temperature was raised by a fraction of a degree and the change in fluorescence was measured. At the melting point, the two strands of DNA would be separated and the fluorescence rapidly decreased. Y-axis shows the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) and X-axis represents the temperature. Peak observed indicates the melting temperature (T_m) of the PCR products.

4.3   Regulation of PPARα expression in response to TNF-α

4.3.1   Dose-response effect on PPARα expression

The effect of TNF-α on PPARα expression is crucial in the maintenance of lipid homeostasis. Therefore, the effect of various concentrations of TNF-α was examined in HepG2 cells, as described in Section 3.3. The outcome of TNF-α on the PPARα mRNA and protein levels is as shown in Figure 4.4. Both PPARα mRNA and protein were normalised with the β-actin expression. Overall, the PPARα mRNA levels and protein contents were observed to decrease steadily in cells treated with increasing concentrations of TNF-α (0 – 100 ng/ml).

As observed in Figure 4.4a, treatment with 20 ng/ml of TNF-α was enough to significantly decrease the PPARα mRNA levels to approximately 0.60-fold. A maximal reduction was observed at 100 ng/ml of TNF-α, whereby the PPARα mRNA was decreased significantly to 0.48-fold relative to the untreated cells (control). Western blot analysis was further carried out in order to determine whether the TNF-α-mediated reduction of PPARα mRNA expression was also observed at the level of the PPARα protein. A protein band with the expected size of approximately 55 kDa, which corresponded to the molecular weight of PPARα protein, was successfully detected (Figure 4.4b). Densitometric analysis indicated that the TNF-α suppressed steady state protein levels of PPARα as compared to untreated control (which was assigned 1.00). On the other hand, β-actin protein levels remained unaffected. PPARα protein level was significantly suppressed to

(a)

(b)
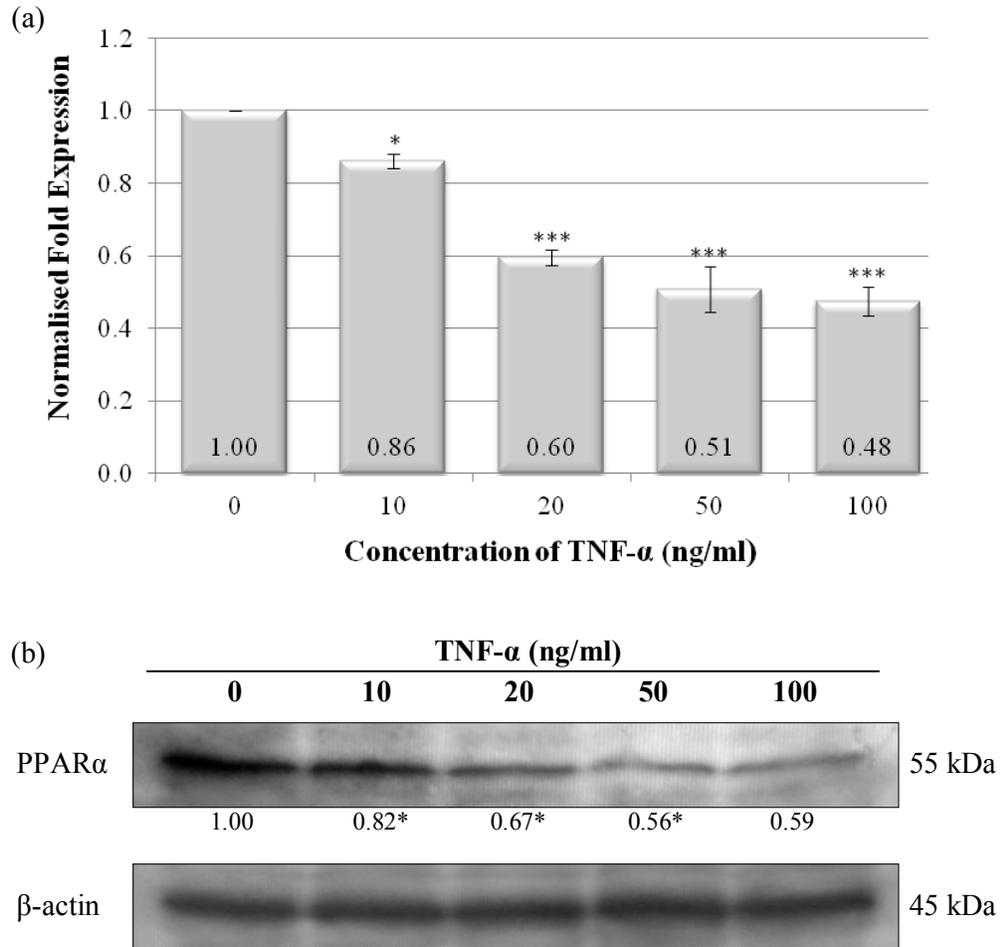
Figure 4.4: Dose-dependent manner of PPARα expression in HepG2 cells stimulated with TNF-α.
HepG2 cells were treated with different concentrations of TNF-α for 24 h before they were harvested for their total mRNA and protein, respectively. (a) Expression profile of PPARα mRNA measured by quantitative Real-Time RT-PCR. Values represent means ± SEM of PPARα mRNA from triplicate of four independent experiments (n=4) normalised to β-actin mRNA, relative to untreated cells values (control). (b) Western blot analyses of PPARα and β-actin. Total cellular proteins (100 μg of protein per lane) were probed against the respective antibodies and detected using secondary HRP-conjugated anti-rabbit IgG antibodies. Values shown below are representation of the relative PPARα protein expression in each treatment towards untreated sample (assigned as 1.00) after normalisation to β-actin. * $P<0.05$ and *** $P<0.001$, significantly different from the respective control group.

approximately 0.82, 0.67, 0.56, and 0.59, in cells treated with 10, 20, 50, and 100 ng/ml of TNF-α, respectively.

4.3.2 PPARα mRNA stability test

Since TNF-α had shown to down-regulate PPARα expression, this have suggested the possibility that PPARα gene could be regulated by the modulation of transcription initiation. Nevertheless, if transcription initiation is not the primary gene regulation for PPARα, an alternative possibility is that the gene could be regulated at the level of mRNA stability instead. Thus, it was worthwhile to determine whether mRNA stability contributed to the decrease of expression. To determine if the mechanism that mediated the inhibitory effect of TNF-α on PPARα mRNA expression level was a result of post-transcriptional mechanism, mRNA stability test was carried out with actinomycin D. HepG2 cells were either left untreated or treated with 20 ng/ml of TNF-α for another 24 h as described in Section 3.4.

Chew (2006) has shown that the incubation period for actinomycin D should be limited to 4 h because further incubation of HepG2 cells with the compound was observed to cause cell death. PPARα mRNA degradation was found to be mostly similar in both cells stimulated in the absence or presence of TNF-α, suggesting that PPARα mRNA was stable (Figure 4.5). This result strongly indicates that TNF-α down-regulatory effect on PPARα mRNA level was mediated primarily at the rate of PPARα gene transcription.
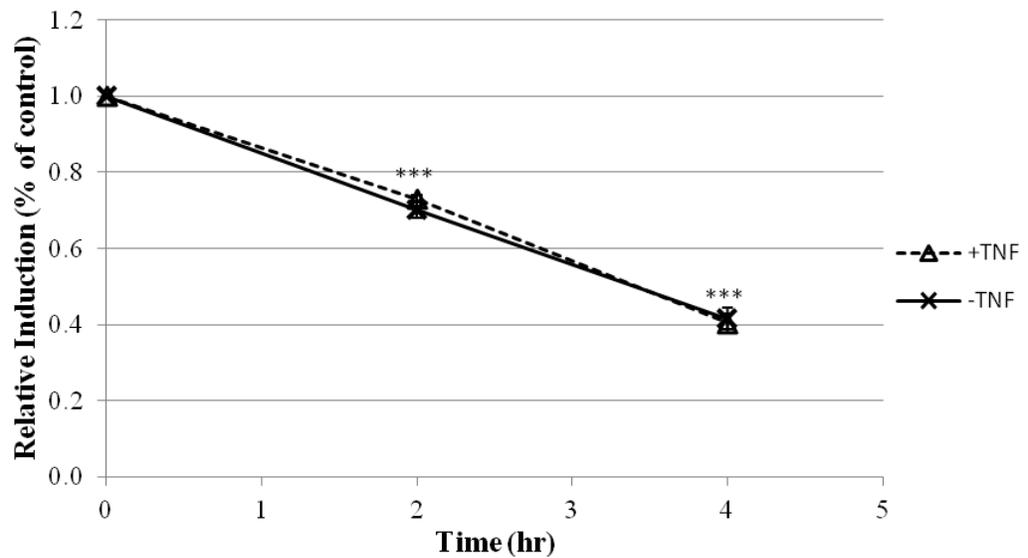
Figure 4.5: Effect of TNF-α on PPARα mRNA stability.
HepG2 cells were stimulated either in the presence (dashed line) or absence (black line) of 20 ng/ml of TNF-α for 24 h. Next, 5 g/ml of actinomycin D was added and incubated for an additional 2 or 4 h. Total cellular RNA was extracted and analysed for PPARα and β-actin mRNA by Real-Time RT-PCR. The results presented here are the average of triplicates of three independent experiments ± SEM (n=3). *** represents $P<0.001$, as compared to their respective control groups.

4.3.3   Time-course studies on PPARα expression

Based on Section 4.3.1, HepG2 cells were exposed to TNF-α for a single fixed period of time. To have further insights into the time-course of the responses, the cells were either left untreated or exposed to a single dose (20 ng/ml) of TNF-α for different lengths of time (1 h, 2 h, 4 h, 8 h, 16 h, and 24 h). As shown in Figure 4.6, PPARα mRNA decreased in a time-dependent fashion after exposure to TNF-α. To ensure that the decrease was mediated in the cells in respond to TNF-α, untreated HepG2 cell controls at each time point were included. PPARα mRNA expression was shown to decrease to 0.96-fold after 1 h of incubation with TNF-α, which was followed by a subsequent decrease to 0.86-fold at 2 h of incubation. Nonetheless, the reduction in PPARα was almost sustained after 4 h of incubation, where the normalised expression was approximately 0.67-fold when compared with the constitutive PPARα mRNA level of unstimulated cells at their respective time. At 24 h, the mRNA was decreased to approximately 0.64-fold, which was not much significantly different from the decrease at 4 h. Therefore, the incubation period of 4 h with 20 ng/ml of TNF-α was chosen for subsequent experiments.

4.4   Effects of serum deprivation on PPARα expression

Prior to all stimulations, the HepG2 cells were pre-incubated in the medium with a reduced concentration of heat-inactivated FBS (0.5% (v/v)) for 2 h, in order to limit the amount of growth factors presence in FBS and to ensure the effect on PPARα mRNA expression was due to the treatment by cytokine. However, because the expression of PPARα may be affected by
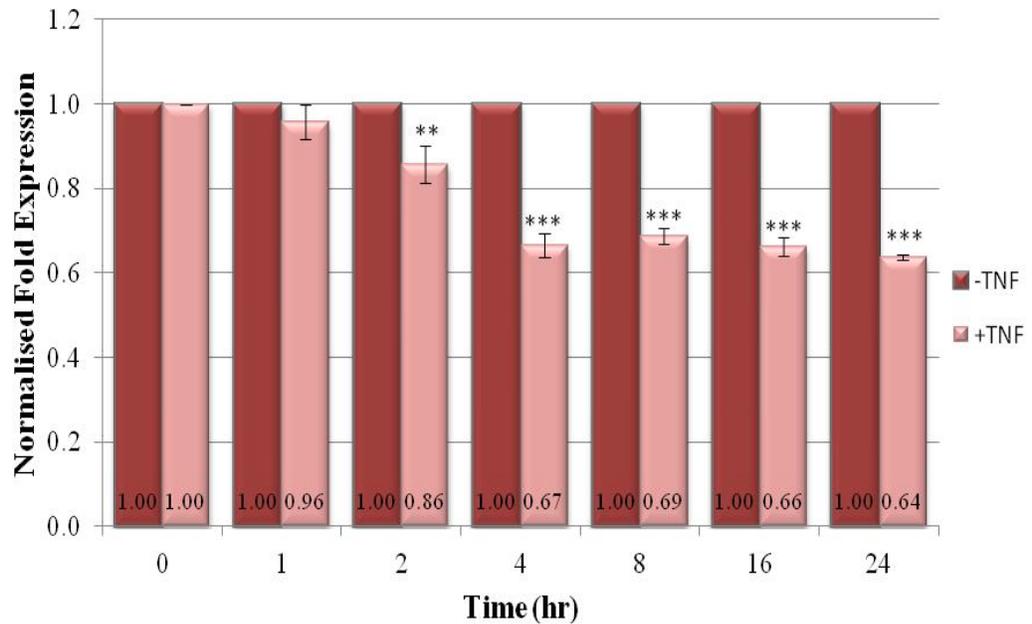
Figure 4.6: Time-dependent appearance of PPARα expression in HepG2 cells stimulated with TNF-α.
PPARα mRNA expression was analysed by Real-Time RT-PCR. The mRNA levels were normalised to β-actin mRNA and then compared with the untreated cells at each time point (assigned as 1.00), before they were expressed in fold-change, relative to time 0 h (untreated cells referred to as control). Values represent means ± SEM of PPARα mRNA from triplicate of three independent experiments (n=3). ** $P<0.01$ and *** $P<0.001$, significantly different from the respective control group.

fasting and starvation (Kersten *et al.*, 1999; van der Lee *et al.*, 2001), PPARα mRNA expression was analysed from the cells with or without the serum starvation step in order to investigate the effects of serum starvation on PPARα levels in this study (Figure 4.7). The experiment showed that there was no significant effect on PPARα expression when the cells were serum starved for 6 h.

4.5 Optimisation of Wedelolactone conditions on PPARα expression

4.5.1 Dose-response effect of the inhibitor

PPARα has been known to pose anti-inflammatory effects, which is mainly achieved by down-regulating pro-inflammatory genes, in which NF-κB is one of the critical pro-inflammatory mediators (Lefebvre *et al.*, 2006). On the other hand, screening of upstream signalling mediators of TNF-α using a panel of inhibitors was conducted in the laboratory by another colleague and the preliminary results showed that IKK inhibitor II (Wedelolactone), a selective and irreversible inhibitor of IKKα and β kinase activity (Kobori *et al.*, 2004), significantly induced expression of PPARα (Appendix C). Thus, this result indicates that the transcriptional interference between PPARα and NF-κB occurs reciprocally.

The duration of signal transduction is crucial and usually happens in a short period of time, so that it can elicit a complex cellular response to maintain the homeostasis in the presence of a sustained stimulus. The mechanisms of feedback regulation that underlie intracellular signalling system usually occur within 2 h as shown by Behar *et al.* (2007). In the

65
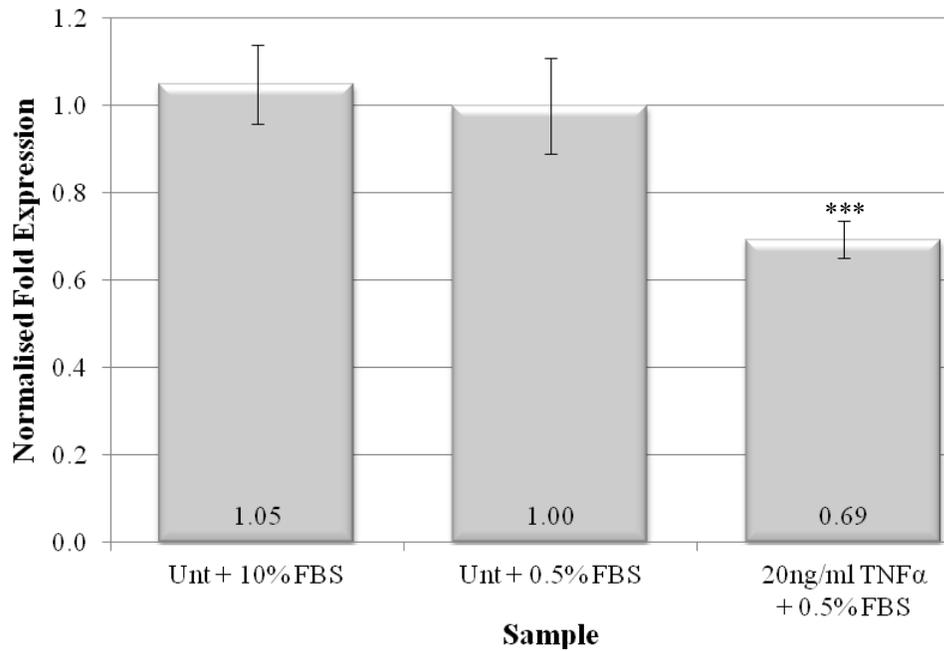
Figure 4.7: PPARα expression in serum-starved HepG2 cells.
HepG2 cells were cultured either in a complete medium (10% (v/v) of FBS) or medium with 0.5% (v/v) concentration of FBS for a total of 6 h. For the positive control, the cells were pre-incubated in medium with a reduced concentration of FBS (0.5% (v/v)) for 2 h prior to TNF-α addition for 4 h. Total cellular RNA was isolated and analysed for PPARα and β-actin mRNA by Real-Time RT-PCR. The results presented here are the average of triplicates of three independent experiments ± SEM (n=3). *** represents $P<0.001$, as compared to the control (which assigned as 1.00).

present study, the determination of the optimum Wedelolactone dosage which can induce PPARα expression was carried out. The cells were pre-incubated with various concentrations of Wedelolactone for a fixed period of 2 h before TNF-α treatment for 4 h (Section 3.5).

Wedelolactone inhibits NF-κB-mediated gene transcription by blocking the phosphorylation and degradation of IκBα (Kobori *et al.*, 2004). In Figure 4.8, cells which were pre-treated with 10 μM of Wedelolactone before TNF-α stimulation showed a significant induction in PPARα expression (1.86-fold) to more than its basal levels (1.47-fold), as compared to cells treated with TNF-α alone (which is assigned as 1.00). Vehicle (DMSO) did not affect the basal levels of PPARα expression (Appendix D). PPARα mRNA levels were subsequently increased gradually to approximately 2.07-fold, but was reduced slightly at 1.95-fold in cells pre-treated with 25 μM and 50 μM of Wedelolactone, respectively. Since a lower concentration of Wedelolactone (10 μM) was sufficient to abrogate the TNF-α repression on PPARα expression to a level higher than its basal levels, this concentration was used for further experiments in this study.

4.5.2 Time-course studies of the inhibitor

According to Section 4.5.1, the HepG2 cells were pre-treated with Wedelolactone for a single fixed period of time. Time-course of the responses was performed to provide insights into the regulation of the gene by the inhibitor. The cells were pre-treated with 10 μM of Wedelolactone for
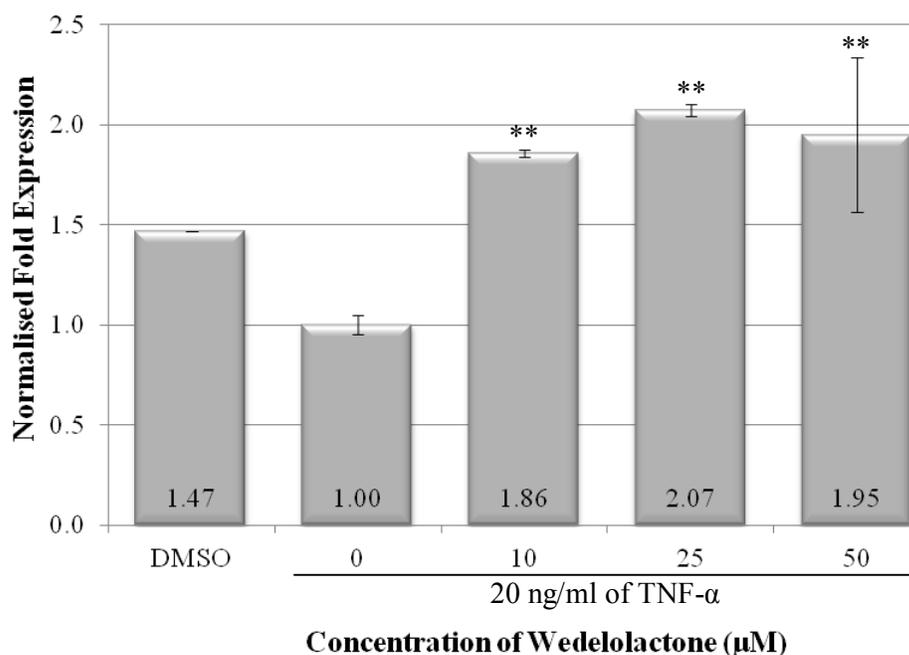
Figure 4.8: Pre-treatment with Wedelolactone attenuated TNF-α down-regulatory action on PPARα expression in dose-dependent manner.
Before 20 ng/ml of TNF-α was added for 4 h, HepG2 cells were pre-stimulated with various concentration of Wedelolactone for 2 h. PPARα and β-actin mRNA were analysed by quantitative Real-Time RT-PCR. Values represent means ± SEM of PPARα mRNA from triplicate of three independent experiments (n=3) normalised to β-actin mRNA, relative to the values of cells stimulated with TNF-α only (control). ** $P<0.01$, significantly different from the respective control group.

different lengths of time (0.5 h, 1 h, 2 h, and 3 h) before the TNF-α treatment for 4 h, which was pre-determined in Section 4.3.3 as the time of incubation enough to cause a significant decrease in PPARα expression. The TNF-α-stimulated cell without pre-treatment with Wedelolactone was assigned as control. This study showed that the exposure of cells to 20 ng/ml of TNF-α after pre-treatment with 10 μM of Wedelolactone resulted in a time-dependent increase in PPARα expression (Figure 4.9). A significant increase (1.68-fold) in PPARα mRNA expression was detected after 2 h pre-incubation and its expression reached constant afterward.

4.6   TNF-α regulates PPARα in a manner dependent on NF-κB activation

Pre-treatment of HepG2 cells with Wedelolactone, which is one of the NF-κB inhibitor, provides the insight into the potential regulation of PPARα expression by NF-κB. Hence, to further confirm whether the repressive effect of TNF-α on PPARα mRNA expression was mediated through activation of NF-κB pathway, two other classes of inhibitors for NF-κB pathway were used in this experiment. They were IKK-2 inhibitor (SC-514) and NF-κB activation inhibitor IV.

Briefy, HepG2 cells were pre-incubated with inhibitors for 2 h before stimulation with 20 ng/ml of TNF-α for 4 h as described in Section 3.5. As shown in Figure 4.10, in cells without any inhibitor pre-treatment, TNF-α stimulation significantly led to a reduction of PPARα mRNA to approximately
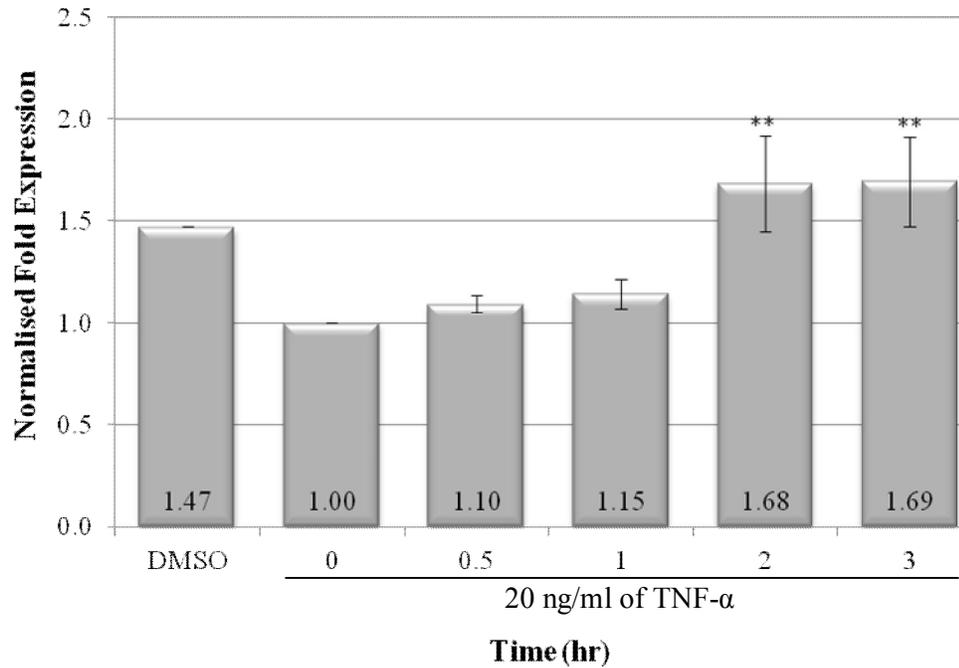
Figure 4.9: Pre-treatment with Wedelolactone attenuated TNF-α down-regulatory action on PPARα expression in time-dependent manner.
HepG2 cells were pre-stimulated with 10 μM of Wedelolactone for various time intervals for time-course study before 20 ng/ml of TNF-α was added for an additional 4 h. Total cellular RNA was isolated from cells and analysed for PPARα and β-actin mRNA by Real-Time RT-PCR. Values represent means ± SEM of PPARα mRNA from triplicates of three independent experiments (n=3) normalised to β-actin mRNA, relative to the values of cells at 0 h time point (control). ** $P<0.01$, significantly different from the respective control group.
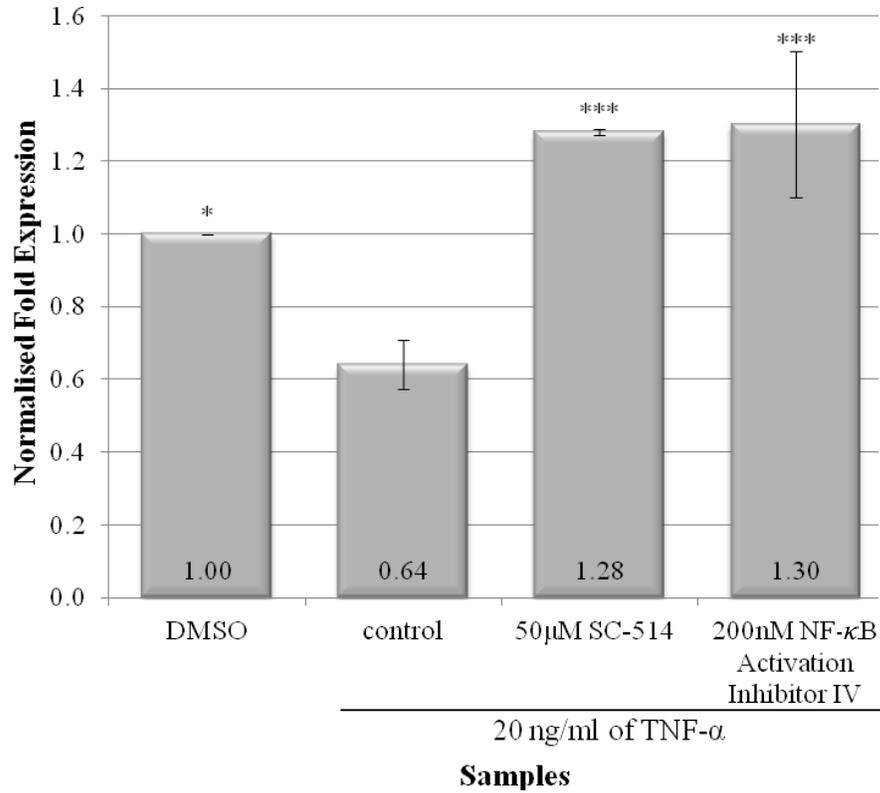
Figure 4.10: TNF-α suppresses PPARα expression through NF-κB signalling pathway.
HepG2 cells were pre-treated with different inhibitors for 2 h before 20 ng/ml of TNF-α was added for an additional 4 h. Total cellular RNA was isolated from cells and PPARα mRNA expression was determined by Real-Time RT-PCR and normalised to β-actin mRNA expression. Values represent means ± SEM of PPARα mRNA from triplicates of three independent experiments (n=3), relative to the values of cells without pre-treatment of any inhibitor (control). * $P<0.05$ and *** $P<0.001$, significantly different from the respective control group.

0.64-fold levels of vehicle-treated cells (assigned as DMSO). Pre-treatment with IKK and NF-κB activation inhibitors prior to TNF-α stimulation resulted in induced PPARα expression to 1.28- and 1.30-fold, respectively. These inductions in expression were significant when compared to TNF-α-stimulated cells without any inhibitor pre-treatment (assigned as control). Collectively, these results demonstrated that TNF-α suppression of the PPARα expression was mediated through the NF-κB signalling pathway.

4.7 Effect of Wedelolactone pre-treatment and TNF-α treatment on the level expression of PPARα target genes

PPARα is an important regulator of cellular fatty acid uptake and intracellular fatty acid transport, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis, and gluconeogenesis (Stott *et al.*, 1995). Recent studies in rodents have suggested that activation of PPARα influences hepatic triglyceride synthesis and cholesterol homeostasis by interacting with gene expression or proteolytic activation of sterol regulatory element-binding proteins (SREBPs) (Knight *et al.*, 2005; König *et al.*, 2007). Previous experiments have demonstrated that Wedelolactone pre-treatment was capable of restoring PPARα expression to more than its basal levels. The effect of Wedelolactone on expression of PPARα target genes in HepG2 cells, however, has not yet been investigated. Thus, the determination of mRNA levels of PPARα target genes (apolipoprotein (Apo) AI and ApoB), which are involved in lipoprotein metabolism, was conducted to examine PPARα activation in this liver model by Wedelolactone.

72

After pre-incubation with 10 µM of Wedelolactone for 2 h, HepG2 cells were stimulated with 20 ng/ml of TNF-α for 4 h (Section 3.5). PPARα target genes, which are ApoAI and ApoB genes expression, were analysed by using Real-Time RT-PCR. Based on Figure 4.11, results obtained for PPARα expression were in accordance with those obtained in Section 4.5. Compared to the controls (assigned as DMSO), TNF-α treatment decreased ApoAI gene expression by 0.67-fold and moderately increased ApoB gene expression to 1.13-fold. Conversely, Wedelolactone pre-treatment after TNF-α stimulation significant induced ApoAI expression to approximately 1.10-fold; while ApoB expression was significant reduced to approximately 0.78-fold, as compared to TNF-α stimulation without Wedelolactone pre-treatment.

4.8 Investigation of the mechanisms of NF-κB signalling pathway

4.8.1 Activation of classical NF-κB signalling pathway abrogated TNF-α down-regulatory effect on PPARα expression

NF-κB activation consists of two separate pathways (Bhatnagar *et al.*, 2010). Although they are often activated concurrently, classical and alternative NF-κB activation pathways have distinct regulatory functions (Nishikori, 2005). Therefore, to elucidate whether TNF-α triggers the classical or alternative NF-κB signalling pathway, the ability of TNF-α to stimulate the processing of p105 to p50 or p100 to p52, and the subsequent p50 and p52 nuclear translocation were studied. By western blot analysis, total p105, p100, p50, and p52 protein levels were determined in nuclear and cytoplasmic extracts from HepG2 cells cultured with TNF-α for the time indicated (Section 3.8.2 and Section 3.8.4). The β-actin and lamin A/C were used as an internal
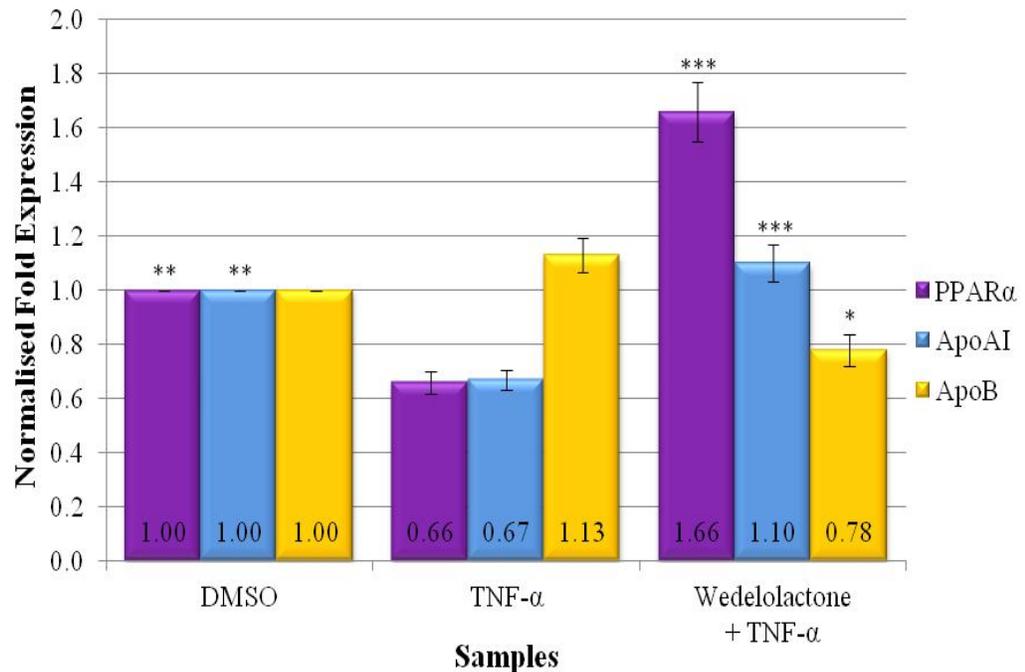
Figure 4.11: Expression of PPARα target genes in TNF-α-stimulated HepG2 cells pre-treated with Wedelolactone.
HepG2 cells were pre-stimulated with 10 μM Wedelolactone for 2 h before 20 ng/ml of TNF-α was added for an additional 4 h. Total cellular RNA was isolated from cells and PPARα, ApoAI, and ApoB mRNA expression were determined by Real-Time RT-PCR and normalised to β-actin mRNA expression. Values represent means ± SEM of mRNAs from triplicates of three independent experiments (n=3), relative to the values of TNF-α treated cells without pre-treatment of Wedelolactone (assigned as TNF-α). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$, significantly different from the respective control group.

loading control to normalise the signal obtained respectively for cytoplasmic and nuclear cell extracts, respectively. Since, β-actin could not be detected in the nuclear fractions of HepG2 cells (data not shown), lamin A/C was used instead.

As shown in Figure 4.12a, cytoplasmic p105, p100, and p52 were shown to be constitutively expressed while expression of p50 was slightly increased in accordance to time. However, the precursor p105 and p100 were undetectable in the nuclear fraction of HepG2 cells (Figure 4.12b). Active p50 and p52 proteins were detected in nuclear fractions of HepG2 cells. The presence of TNF-α did not show statistically significant differences for the nuclear p52 levels throughout 4 h. Nonetheless, a marked nuclear translocation of active p50 was observed following stimulation with TNF-α in 15 min, and significant p50 levels in the nuclear were further increased up to 4 h. Taken together, these results suggest that TNF-α could repress the PPARα expression through augmenting the classical NF-κB signalling pathway and increasing the translocation of p50 into the nucleus.

4.8.2   IKK downstream kinases, but not IKK, are affected by Wedelolactone treatment

Furthermore, in order to elucidate possible signalling mechanism that lead to PPARα reduction in TNF-α-stimulated cells, the changes in the level of protein kinases were examined by western blot analysis (Section 3.8.4). Notably, a large group of protein kinases has been shown to stimulate NF-κB
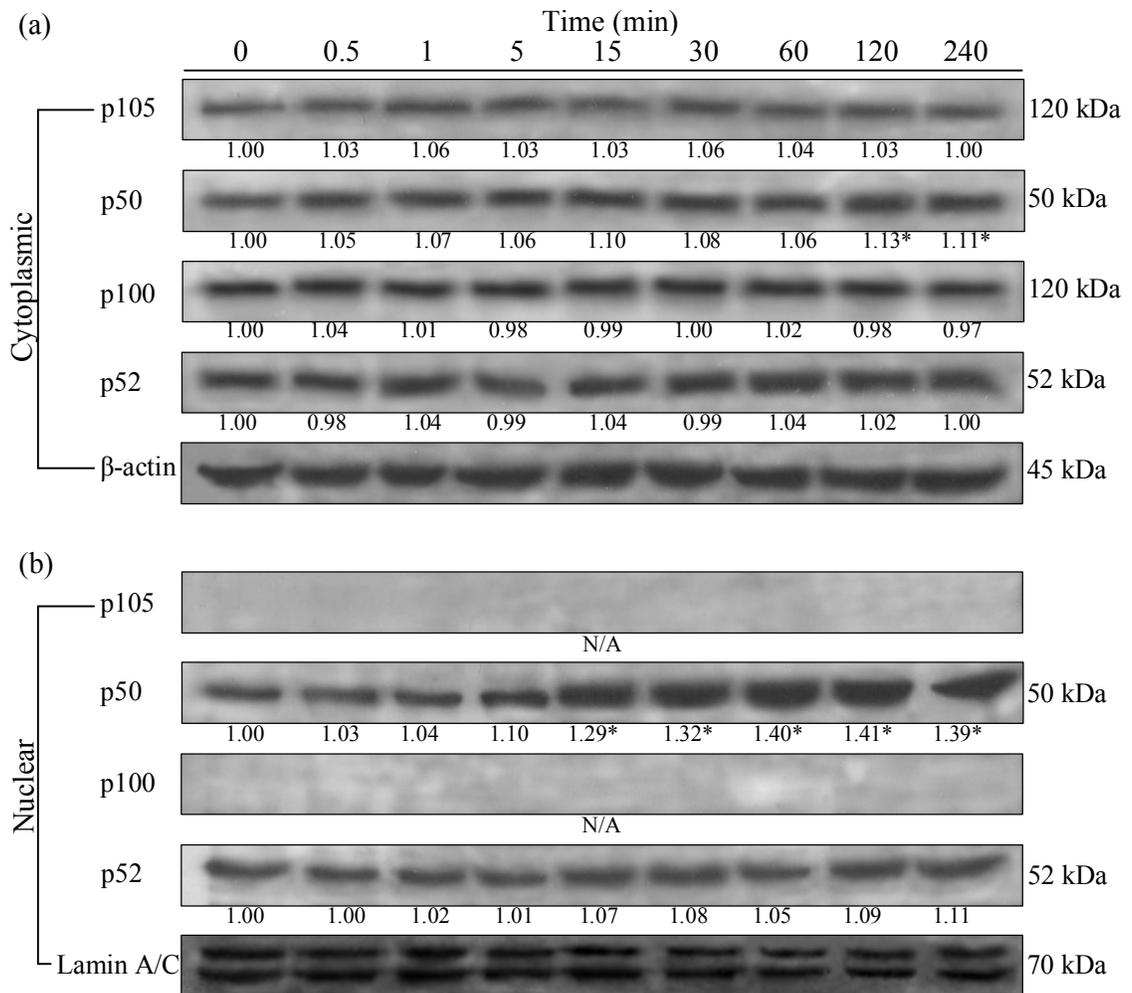
Figure 4.12: Representative western blots for nuclear translocalisation of p50 and p52 in TNF-α-stimulated HepG2 cells.

HepG2 cells were stimulated with 20 ng/ml of TNF-α for the indicated times. At the end of the incubations, (a) cytoplasmic and (b) nuclear fractions were prepared and subjected to immunoblot analysis with anti-NF-κB p105/p50 and anti- NF-κB2 p100/p52 antibodies. For quantification, the band density of each band was normalised their respective loading control. β-actin and lamin A/C antibodies were analysed as loading controls for cytoplasmic and nuclear fractions, respectively. Time at 0 min indicates unstimulated control. This is a representation of three independent experiments (n=3). N/A indicates not available. * $P<0.05$, significantly different from the respective control group.

activity under different conditions. However, the IKK kinase complex seems to be the core element of NF-κB cascade (Israël, 2010) and it has been implicated in the phosphorylation of several IκB proteins and NF-κB family proteins (Gilmore, 2006).

In the present study, IKKα expression for all samples were constant 4 h after TNF-α stimulation (Figure 4.13a), regardless of Wedelolactone pre-treatment. Phosphorylation of IKKα/β at serine 180/181 was significantly increased to approximately 1.51-fold and 1.57-fold after 4 h of TNF-α stimulation in Wedelolactone-untreated and -treated cells, respectively (Figure 4.13b). Based on Figures 4.13c and 4.13e, TNF-α treatment caused significant reductions in both IκBα and IκBβ total protein levels, but enhanced the level of phosphorylation in IκBα at Ser32 and IκBβ at Thr19/Ser23 to 2.13-fold and 2.78-fold (Figures 4.13d and 4.13f), respectively. This could be consequence of IKK-mediated, phophorylation-induced degradation of IκBs, as compared with unstimulated cells. On the contrary, treatment of HepG2 cells with Wedelolactone prior to TNF-α addition showed no effects on total IκBα and IκBβ proteins level (Figure 4.13c and 4.13e), even though their expression was lowered compared to the Wedelolactone-untreated cells. On the other hand, phosphorylated IκBα and IκBβ were almost abolished and were slightly restored after 4 h of TNF-α stimulation (Figure 4.13d and 4.13f). Remarkably, the expression of these two kinases is dependent on IKK activity, as Wedeloloctone blocks the IKKα and β activity.
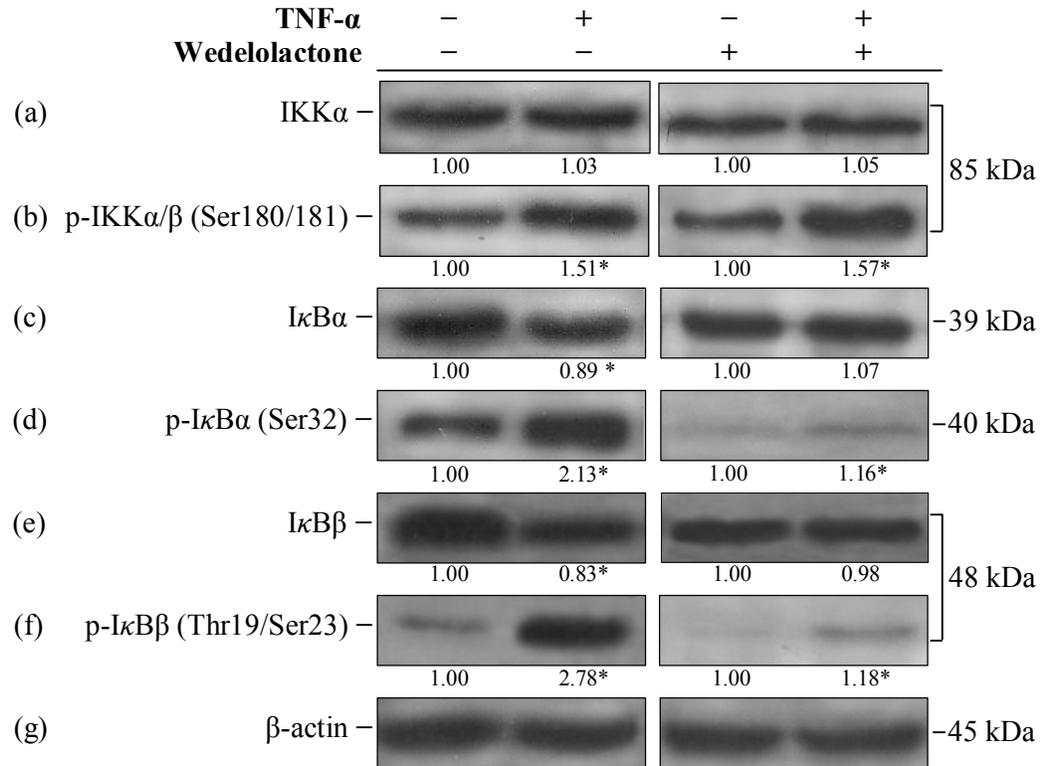
Figure 4.13: Representative western blots for (a) IKKα, (b) p-IKKα/β, (c) IκBα, (d) p-IκBα, (e) IκBβ, (f) p-IκBβ, and (g) β-actin expression in cytoplasmic fractions from HepG2 cells.
HepG2 cells were pre-treated with or without 10 μM of Wedelolactone for 2 h before addition of TNF-α (20 ng/ml) for 4 h. Expression of indicated proteins were analysed by immunoblotting against their specific antibodies. For quantification, the densitiy of each band was normalised against β-actin expression. This is a representation of three independent experiments (n=3). * $P<0.05$, significantly different from the respective control group.

4.8.3 IKK mediates phosphorylation of p105 and p100

The activation of NF-κB dimers is subjected to the degradation of IκB inhibitors, which enable the NF-κB dimers to enter the nucleus and activate specific target gene expression. Mohamed *et al.* (2009) reviewed that the p105 and p100 proteins function both as cytosolic IκB proteins and as precursors of the NF-κB subunits p50 and p52, respectively. Thus, the effects of Wedelolactone on the phosphorylation of NF-κB family proteins were examined.

As shown in Figure 4.14a, p105 expression was constant in all of the samples. However, phosphorylated p105 at Ser933 proteins, which was induced with TNF-α was almost undetectable in samples pre-treated with Wedelolactone, as compared with the samples which stimulated with TNF-α only (Figure 4.14b). Surprisingly, Wedelolactone pre-treatment did not affect the expression levels of TNF-α-induced phosphorylated-p100 at Ser866 or Ser870 (Figure 4.14e). As the degradation or processing of p105 and p100 generated the subunits p50 and p52, respectively, their expression levels were also evaluated. In TNF-α-stimulated cells, p50 expression was significantly increased to 1.28-fold, as compared with the unstimulated cells, but remained constant in the presence of Wedelolactone (Figure 4.14c). This suggests that processing of p105 to p50 was triggered by TNF-α treatment but was abolished in the presence of Wedelolactone. There was no increase in p52 expression in TNF-α-treated cells compared to TNF-α-untreated cells (Figure 4.14f). However, upon Wedelolactone pre-treatment, p52 expression levels were relatively lower (Figure 4.14f); while p100 expression levels

	TNF-α	−	+	−	+
	Wedelolactone	−	−	+	+

(a)	p105 −				−120 kDa
		1.00	1.07	1.00	0.96
(b)	p-p105 (Ser933) −				−120 kDa
		1.00	1.11*	1.00	1.08
(c)	p50 −				−50 kDa
		1.00	1.28*	1.00	0.99
(d)	p100 −				−120 kDa
		1.00	1.05	1.00	1.09
(e) p-p100 (Ser866/870) −					−110 kDa
		1.00	1.14*	1.00	1.05
(f)	p52 −				−52 kDa
		1.00	1.02	1.00	1.04
(g)	β-actin −				−45 kDa
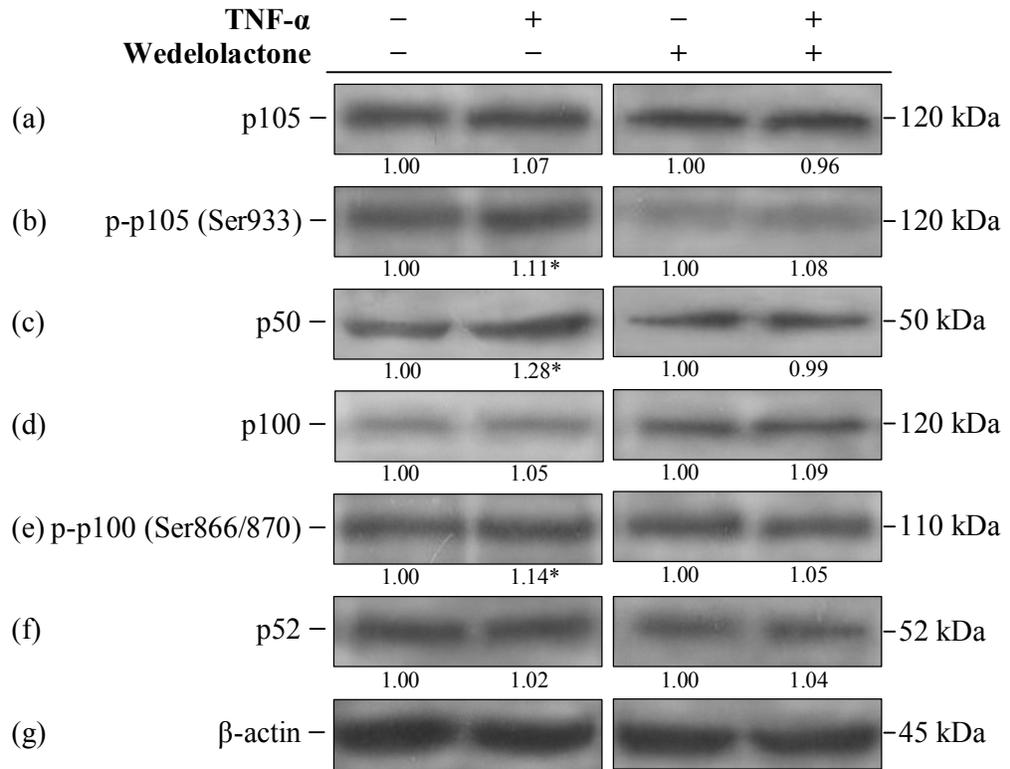
Figure 4.14: Representative western blots for (a) p105, (b) p-p105, (c) p50, (d) p100, (e) p-p100, (f) p52, and (g) β-actin expression in cytoplasmic fractions from HepG2 cells.
HepG2 cells were pre-treated with or without 10 µM of Wedelolactone for 2 h before addition of TNF-α (20 ng/ml) for 4 h. Expression of indicated proteins were analysed by immunoblotting against their specific antibodies. For quantification, the densitiy of each band was normalised against β-actin expression. This is a representation of three independent experiments (n=3). * $P<0.05$, significantly different from the respective control group.

were relatively higher (Figure 4.14d), as compared to respective Wedelolactone-untreated cells. These results coincide with reports describing that IKK complex promotes the ubiquitination of p100 and the proteasomal processing of the complex (Derudder *et al.*, 2003; Bonizzi *et al.*, 2004).

4.8.4 Wedelolactone pre-treatment abrogates Ser468- and Ser536-phosphorylated p65 in TNF-α-stimulated cells

NF-κB is activated by phosphorylation, which plays a key role in the regulation of its transcriptional activity, and is associated with nuclear translocation, CBP recruitment and DNA-binding activity (Reber *et al.*, 2009). Owing to its abundance in most cell types and the presence of a strong transactivational domain, p65 (also known as RelA) is responsible for most of NF-κB's transcriptional activity through its phosphorylation at multiple serine residues (Hu *et al.*, 2004; Reber *et al.*, 2009). Hence, this experiment was conducted to elucidate the phosphorylation sites of p65 which could be involved in the suppression of PPARα expression by TNF-α.

Based on Figure 4.15a, total p65 was shown to be expressed in both Wedelolactone-untreated and Wedelolactone-treated cells. In untreated cells (without TNF-α and Wedelolactone treatments), p65 was constitutively expressed through its phosphorylation sites at Ser276, Ser468, and Ser536 (Figure 4.15b – d). This is in accordance with the biological role of NF-κB family in the development and maintenance of cells and tissues (Hayden and Ghosh, 2011). Subsequent stimulation with TNF-α significantly induced the phosphorylation of p65 at Ser276, Ser468, and Ser536 sites to approximately
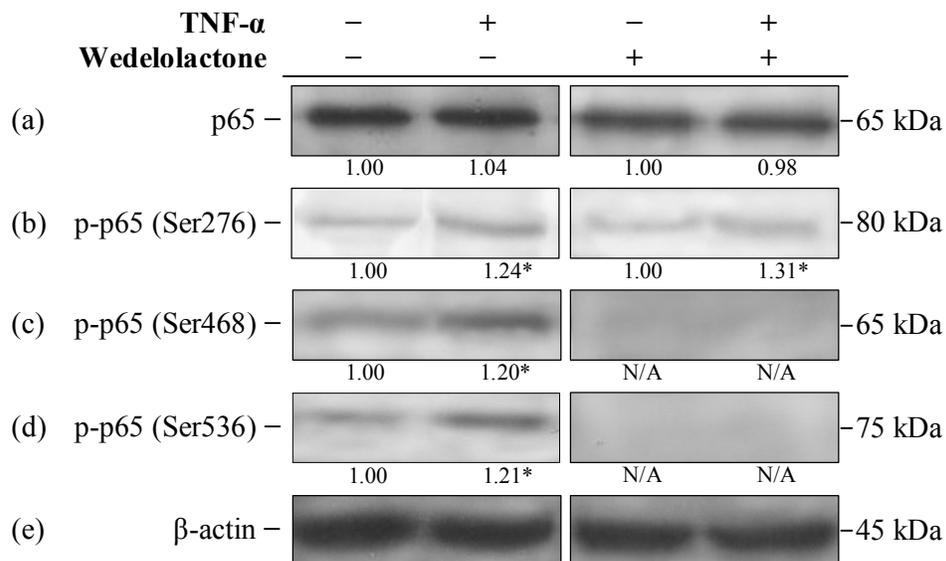
Figure 4.15: Representative western blots for (a) p65, (b) p-p65 (Ser276), (c) p-p65 (Ser468), (d) p-p65 (Ser536), and (e) β-actin expression in cytoplasmic fractions from HepG2 cells.
HepG2 cells were pre-treated with or without 10 μM of Wedelolactone for 2 h before addition of TNF-α (20 ng/ml) for 4 h. Expression of indicated proteins were analysed by immunoblotting against their specific antibodies. For quantification, the densitiy of each band was normalised against β-actin expression. This is a representation of three independent experiments (n=3). N/A indicates not available. * $P<0.05$, significantly different from the respective control group.

1.24-, 1.20-, and 1.21-fold, respectively (Figure 4.15b – d). In response to Wedelolactone pre-treatment, the inhibitor totally abolished the p65 phosphorylation at Ser468 and Ser536 (Figure 4.15c and d), but no effect was observed on Ser276 phosphorylation (Figure 4.15b). Subsequent TNF-α treatment also failed to restore the phosphorylation for both of the Ser468 and Ser536 residues in Wedelolactone-treated cells. It is therefore postulated that these two phosphorylation sites (Ser468 and Ser536) of p65 are dependent on IKK activation in HepG2 cells and may be involved in mediating TNF-α down-regulation on PPARα expression.

4.8.5   Phosphorylated-p65 is translocated into the nuclear fraction

Nuclear translocation of NF-κB is required for the induction or repression of transcription of target genes. As shown in previous experiments, there was an activation of p65 in TNF-α-treated cells. To further investigate whether the activated p65 was translocated into the nuclear fraction, the nuclear extracts of HepG2 cells were subjected to immonoblotting as described in Section 3.8.4.

According to Figure 4.16a, p65 protein was significantly increased to 1.23-fold in response to TNF-α in the nuclear fractions. However, in Wedelolactone-treated cells, p65 total protein was expressed at relatively lower levels than in Wedelolactone-untreated cells. Phosphorylation of p65 at Ser276, Ser468, and Ser536 was shown to be induced in the nuclear fractions of TNF-α-stimulated cells, whereas only phosphorylation of Ser468 and Ser536 was severely abrogated in the presence of Wedelolactone in the

| | TNF-α | − | + | − | + |
| | Wedelolactone | − | − | + | + |

(a) p65 − | 65 kDa
1.00 | 1.23* | 1.00 | 1.05*

(b) p-p65 (Ser276) − | 80 kDa
1.00 | 1.17* | 1.00 | 1.11*

(c) p-p65 (Ser468) − | 65 kDa
1.00 | 1.18* | N/A | N/A

(d) p-p65 (Ser536) − | 75 kDa
1.00 | 1.13* | N/A | N/A

(e) lamin A/C − | 70 kDa
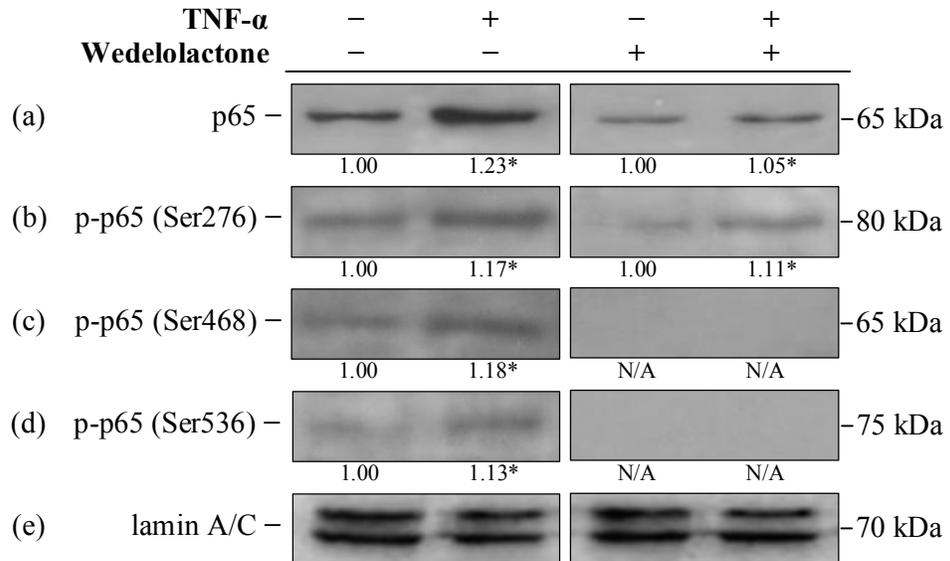
Figure 4.16: Representative western blots for (a) p65, (b) p-p65 (Ser276), (c) p-p65 (Ser468), (d) p-p65 (Ser536), and (e) laminA/C expression in nuclear fractions from HepG2 cells.
HepG2 cells were pre-treated with or without 10 μM of Wedelolactone for 2 h before addition of TNF-α (20 ng/ml) for 4 h. Expression of indicated proteins were analysed by immunoblotting against their specific antibodies. For quantification, the densitiy of each band was normalised against laminA/C expression. This is a representation of three independent experiments (n=3). N/A indicates not available. * $P<0.05$, significantly different from the respective control group.

nuclear fractions (Figure 4.16b – d). This implicated that in the nucleus, phosphorylated p65 proteins had a similar response to the cytoplasmic proteins shown in Figure 4.15 after the Wedelolactone pre-treatment and TNF-α treatment. These results strengthen the evidence in which the phosphorylation sites of p65 at Ser468 and Ser536 might play a key role in the TNF-α down-regulatory effect on PPARα expression, most likely via the activation of IKK/p105/p50/p65 pathway.

CHAPTER 5

DISCUSSION

5.1   HepG2 cells as model system

Inflammation is a critical homeostatic response to a number of injurious stimuli such as damaged cells or pathogens. While it is known that liver plays a key role in the whole body energy homeostasis by its ability to metabolise glucose and fatty acids (Reddy and Rao, 2006), it is also a central regulator of inflammation by its ability to secrete a number of proteins that regulate systemic inflammatory response as well as being the target of inflammatory responses (Dasarathy, 2008). However, during liver inflammation, excessive exposure to the cytokines will eventually cause hepatocyte cell death, which can contribute to acute and chronic liver injuries. PPARα is highly expressed in liver. It plays critical roles in the regulation of lipid metabolism and homeostasis and modulation of inflammatory responses (Desvergne et al., 2004). Hence, human hepatocarcinoma HepG2 cell line was chosen for this study.

Due to their high degree of morphological and functional differentiation similarity to normal hepatocytes, HepG2 cell is an adequate model for many mechanistic studies of signal transduction or intracellular trafficking, gene expression, metabolism and toxicology (Mersch-Sundermann et al., 2004; Saad et al., 2005). On the other hand, it is capable of expressing multiple cytokine genes such as TNF-α, transforming growth factor-β (TGF-β),

macrophage colony-stimulating factor (M-CSF), and interleukin (IL)-receptor (Stonans *et al.*, 1999). This can be important for the study of human liver diseases that are caused by an incorrect subcellular distribution of cell surface proteins, understanding hepatocarcinogenesis, and for drug targeting studies. Besides, HepG2 cells are also able to retain many of the functions of normal hepatocytes such as the synthesis of albumin, lipoproteins, and other liver-specific proteins (Rash *et al.*, 1981; Wang *et al.*, 1988; Javitt, 1990; Hirayama *et al.*, 1993), and therefore, encourages an even wider application of this cell line to biological problems that relate specifically to the role of the liver.

5.2 PPARα gene expression is TNF-α dose- and time-dependent

TNF-α is a multi-functional cytokine that can regulate many cellular and biological processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism (Cawthorn and Sethi, 2008; Hwang *et al.*, 2009). TNF receptor (TNFR) recruits adaptor proteins and leads to the activation of the downstream signal cascade (Aggarwal, 2003). Herein, this current study was to examine systematically the effects of TNF-α on the expression of PPARα mRNA and protein level.

In this study, the suppressive effect of TNF-α on PPARα expression in hepatocytes was observed in a dose- and time-dependent manner (Figure 4.4 and Figure 4.6). These findings are consistent with previous studies which showed that the TNF-α treatment attenuated PPARα gene expression *in vivo* and *in vitro* (Glosli *et al.*, 2005; Kim *et al.*, 2007; Becker *et*

al., 2008). In addition, a similar pattern was also observed in PPARα protein level when cells were stimulated with various concentrations of TNF-α.

TNF-α is a cytotoxic protein. However, unlike some other members of the TNF family such as TNF-related apoptosis-inducing ligand (TRAIL), TNF-α is at most weakly cytotoxic or cytostatic to malignant cells (Balkwill, 2009). It has been reported that TNF-α by itself had virtually no effect on the growth of lung carcinoma cells (Berman *et al.*, 2002), vascular smooth muscle cells (Geng *et al.*, 1996), and hepatocytes (Leist *et al.*, 1994). In agreement with the reports of other studies, untreated cells or cells exposed to 20 ng/ml of TNF-α alone exhibited no morphological signs of apoptosis when analysed by inverted microscopy (data not shown). TNF-α alone could actually induce tolerance to cytotoxicity conditions (Hill *et al.*, 1995; Saas *et al.*, 2005; Saas *et al.*, 2007). Pre-treatment with TNF-α alone, without transcriptionl inhibition, for 2 h to at least 24 h protects mice from apoptotic liver damage subsequently induced by GalN/TNF application, in a study by Wallach *et al.* (1988) and Goto *et al.* (1997).

As a mediator of inflammation, a range from 0 to 100 ng/ml of TNF-α used in this study in accordance with other experimental studies which demonstrated its consequences in fibroblast (Chen and Thibeault, 2010), HepG2 cells (Haas *et al.*, 2003), and eosinophils (Uings *et al.*, 2005). According to the results obtained in Section 4.3.1, PPARα mRNA and protein expression decreased significantly when stimulated with 20 ng/ml of TNF-α and with a maximal effect at 100 ng/ml of TNF-α. Although 50 ng/ml of TNF-

α was shown to reduce PPARα expression significantly, it was not significantly lower than 20 ng/ml of TNF-α. TNF-α incubation time of 4 h was chosen for the subsequent experiments due to the sustained reduction in expression of PPARα as illustrated in Figure 4.6.

5.3 PPARα expression is not affected by serum-deprivation

Serum is a very complex supplement containing mostly proteins but also growth factors, hormones, amino acids, sugars, trypsin inhibitors, and lipids (Adkins *et al.*, 2002). Indeed, the presence of certain general animal-serum components in variable amounts in FBS, such as immunoglobulins, transcription factors, and growth factors, can intervene with the cellular function, growth, and the phenotypic/genotypic stability of the cultured cells (Jochems *et al.*, 2002; Tekkatte *et al.*, 2011). For example, a study by Gu *et al.* (2009) showed that FBS rapidly altered the gene expression of retinal progenitor cells. Hence, HepG2 cells were pre-incubated in the serum-deprived medium for 2 h prior to all stimulation in this study. Similar studies have also used this approach (Wang *et al.*, 2008; Brown *et al.*, 2011). Heat inactivation was also performed to inactivate complement which can lead to complement-mediated cell lysis. Besides, serum starvation could also be used to synchronise the cells (Yan and Tao, 2007; Khammanit *et al.*, 2008). It could reset the cell cycle by causing stress to the cells which then leads to most circulating cells to enter into G0 phase.

However, PPARα which is activated by free fatty acids, plays a crucial role in coordinating metabolic changes caused by fasting and starvation

(Tacer *et al.*, 2007). It has been clearly established that fasting led to a marked rise in PPARα mRNA expression (Kersten *et al.*, 1999; van der Lee *et al.*, 2001; Chakravarthy *et al.*, 2007). PPARα is dominant in mediating the effects of dietary fatty acids on gene expression in liver (Sanderson *et al.*, 2008). Physiological experiments using PPARα$^{-/-}$ mice have shown that PPARα is especially important for the adaptive response to fasting. It governs the fasting-induced up-regulation of numerous genes involved in hepatic fatty acid oxidation, many of which are direct PPARα target genes (Kersten *et al.*, 1999; Hashimoto *et al.*, 2000). Thus, the effect of serum deprivation on PPARα expression was carried out. According to Figure 4.7, PPARα expression did not differ significantly between HepG2 cells that were cultured in complete growth medium or serum-starved medium. This result ensured that the changes in its expression were due to the treatments.

5.4 TNF-α inhibits PPARα gene expression at the transcriptional level

The mRNA levels are determined not only by transcription rates but also by degradation rates. Hence, mRNA stability is another important step in modulating gene expression, in particular for transiently expressed genes that require tightly controlled mRNA levels (Frevel *et al.*, 2003; Molin *et al.*, 2009). In order to test for the mRNA stability, actinomycin D is primarily used as an investigative tool to inhibit transcription. Actinomycin D readily enters most cells, intercalates into DNA, and rapidly blocks transcription and DNA replication (Blattner *et al.*, 1999).

Hill *et al.* (1995) reviewed that HepG2 cells are normally insensitive to TNF-α cytotoxicity, but they are rendered susceptible, or sensitised, to TNF-α cytotoxicity by inhibitors of RNA and protein synthesis. There were also reports which revealed that TNF-α cytotoxic potential is unmasked during co-treatment of TNF-α and metabolic inhibitors (actinomycin D, cyclohexamide or mitomycin) (Dealtry *et al.*, 1987; Pierce *et al.*, 2000) or less specific drugs that also inhibit gene transcription (galactosamine or α-amanitin) (Leist *et al.*, 1998). As a result, the default cells survival and inflammatory pathways downstream of TNF-α signalling are inactivated thus allowing apoptosis to proceed. Moreover, studies conducted on cultured AML12 hepatocytes by Pierce *et al.* (2000) showed that the cells underwent apoptosis when exposed to TNF-α in the presence of actinomycin D. Thus, it was suggested that actinomycin D sensitises cells to TNF-α-induced apoptosis by inhibiting the expression of anti-apoptotic genes (Xu *et al.*, 1998).

In this study, HepG2 cells were treated with 5 µg/ml of actinomycin D due to studies done by Wang *et al.* (2005) and 't Hoen *et al.* (2011) showing that this concentration was deemed optimal to arrest the transcription machinery without inducing apoptosis in cells. In addition, the incubation period of HepG2 cells for actinomycin D was limited to 4 h to prevent any further cell death in these cells (Chew *et al.*, 2006). According to Figure 4.5, TNF-α-mediated reduction of PPARα expression was found to be mediated at the transcriptional level and not at the level of mRNA stability.

5.5   TNF-α-mediated   suppression   of   PPARα   is   abrogated   by Wedelolactone pre-treatment

As a pleiotropic pro-inflammatory cytokine, TNF-α exerts multiple biological effects in different cells, tissues, organs, and species (Chen *et al.*, 2009). The molecular mechanisms underlying these actions are complex and several transduction pathways might be involved. Since earlier experiments showed that TNF-α inhibition of PPARα expression was concomitant with the decrease in the levels of specific protein (Section 5.2), this suggests that the effects of TNF-α may have been exerted on the mechanism which regulates the transcription of PPARα. In order to examine further, an experiment was carried out to elucidate the possible signalling networks that cause the changes in the PPARα expression in response to TNF-α treatment in HepG2 cell line.

NF-κB regulates the activities of many signalling pathways in the intracellular signalling network, thereby playing a critical role in determining cellular responses to extracellular stimuli (Karin and Lin, 2002; Lin, 2003). The preliminary results from predecessors showed that IKK inhibitor II (Wedelolactone) plays a major role in TNF-α signalling on PPARα (Appendix C). It is noteworthy that the endogenous level of PPARα is controlled by multiple important machineries, among which NF-κB pathway is a pivotal one. Therefore, it seems reasonable to hypothesise that targeting the NF-κB pathway may provide insights into the mechanisms of TNF-α-mediated suppression of PPARα. Wajant and co-reseachers (2003) have shown that the transduction pathways activated by TNF-α include mitogen-activated protein kinases (MAPKs) and IKK, which control gene expression through

transcriptional factors such as activator protein-1 (AP-1) and NF-κB. It is also known that TNF-α has stimulatory effects on hepatocytes to produce acute phase reactants through an NF-κB-dependent mechanism (Tian and Brasier, 2003).

Furthermore, there is substantial evidence to show that PPARα often has intracellular interaction with NF-κB, whose activities are mainly in the hepatic acute-phase response, pro-inflammatory and proliferative (Tian and Brasier, 2003). Previous works have reported that PPARs activation suppressed NF-κB activity (Jee *et al.*, 2005; Sasaki *et al.*, 2005a); whereas activation of NF-κB inhibits the transcriptional activity of PPARs (Bumrungpert *et al.*, 2009). Hence, NF-κB and PPARs can also trans-repress each other's activity (Tham *et al.*, 2002; Planavila *et al.*, 2005b). Notably, these findings have conclusively shown that it is possible that NF-κB would be one of the important intermediates in the signalling cascade which represses PPARα mRNA expression when exposed to TNF-α.

To optimise the conditions of NF-κB inhibition that affect the induction of PPARα, the HepG2 cells were treated with Wedelolactone, an inhibitor for IKKα and β activity (Section 3.5). Based on Figure 4.8, a preliminary dose-response in this study demonstrated that PPARα expression was restored to more than its basal levels after TNF-α stimulation at a low concentration (10 µM) of Wedelolactone-treated cells. This result, along with the data presented in Figure 4.9, demonstrate that the expression level of PPARα was elevated in a time-dependent manner in cells pre-treated with

Wedelolactone, which implicates that TNF-α regulates PPARα in a manner dependent on NF-κB activation.

5.6   Inhibition of NF-κB activation attenuates TNF-α down-regulation of PPARα

To further confirm whether PPARα expression is affected by NF-κB activation, apart from Wedelolactone, two disparate inhibitors that block distinct steps in the NF-κB pathway, namely SC-514 and NF-κB activation inhibitor IV were chosen to be tested on HepG2 cells, together with TNF-α stimulation. Since IKK is the converging point for the activation of NF-κB, an inhibitor of IKKβ, SC-514 was used to block the upstream kinase of NF-κB. It inhibits the native IKK complex or recombinant human IKKα/IKKβ heterodimer and IKKβ homodimer similary (Kishore *et al.*, 2003). On the other hand, NF-κB activation inhibitor IV, a cell-permeable resveratrol analog that exhibits enhanced anti-inflammatory potency, was used to inhibit the TNF-α activation of NF-κB.

It is well known that inhibition of NF-κB activation sensitises cells to TNF-induced apoptosis (van Antwerp *et al.*, 1998; Doi *et al.*, 1999; Rosenfeld *et al.*, 2000). However, the HepG2 cells in this study exhibited no morphological signs of apoptosis when monitored visually under an inverted microscope (data not shown), ensuring that the inhibitory actions of these compounds were not attributable to cell death. Moreover, SC-514 (1 – 100 μM) has been shown to cause no cytotoxicity at any of the concentrations examined, as reviewed in a study of cell viability assays by Phulwani *et al.* (2008). Based

on Figure 4.10, both SC-514 and NF-κB activation inhibitor IV significantly induced expression level of PPARα in TNF-α stimulated cells over its baseline, if compared to the untreated cells. The data obtained using these two NF-κB inhibitors showed a similar result with the previous studies using Wedelolactone, confirming the notion that inhibition of TNF-α-induced NF-κB activation attenuated the repression of PPARα expression.

Both Wedelolactone and NF-κB activation inhibitor IV are resveratrol analogs. Results obtained here were in accord with the findings of other investigators that proved that resveratrol inhibits TNF-α-induced NF-κB activation (Csiszar *et al.*, 2006) and is a selective activator of PPARα by endothelial cell-based transfection assay (Inoue *et al.*, 2003). Likewise, Cheng *et al.* (2009) also concluded that resveratrol could up-regulate the expression of PPARα in neurons. However, there was also study which revealed that there was a delay but not a complete blockade in IκBα phosphorylation and degradation in cells pre-treated with SC-514 (Kishore *et al.*, 2003). This may be because this particular inhibitor does not block IKKα activity; the IκBα may still be phosphorylated by this IKK isoform. In this study, no complete blockade in IκBα phosphorylation in Wedelolactone-treated cells after TNF-α stimulation could be due to the low concentration of Wedelolactone used.

5.7   Effect of Wedelolactone pre-treatment on the expression of PPARα target genes

PPARα governs biological processes by altering the expression of a large number of target genes. Accordingly, the specific role of PPARα is

directly related to the biological function of its target genes. Since earlier experiments have shown that Wedelolactone pre-treatment was able to restore PPARα expression to more than its basal level (Section 4.5), the effect of Wedelolactone on PPARα target genes was also examined.

Plasma lipoproteins are responsible for carrying triglycerides and cholesterol in the blood and ensuring their delivery to target organs (Rakhshandehroo *et al.*, 2010). At the molecular level, PPARα agonists raise plasma high-density lipoprotein (HDL) levels in humans, most likely via species-specific mRNA induction of apolipoprotein A-I (ApoAI) and A-II (ApoAII) (Roglans *et al.*, 2002). In addition, PPARα activation by fibrates influences metabolism of both HDL and apolipoprotein B (ApoB)-containing lipoproteins (such as large very low-density lipoprotein (VLDL) particles) in rodents and humans (Peters *et al.*, 1997; Milosavljevic *et al.*, 2001). Hence, PPARα plays an important role in the control of lipoprotein metabolism, with ApoAI and ApoB being the PPARα target genes.

Consistent with earlier reports, the current study showed that TNF-α was able to reduce and induce the ApoAI and ApoB mRNA expression in hepatocytes, respectively (Figure 4.11) (Bartolomé *et al.*, 2007; Qin *et al.*, 2008; Mogilenko *et al.*, 2009; Orlov *et al.*, 2010). Haas *et al.* (2003) revealed that ApoAI mRNA levels were suppressed 50.8% in HepG2 cells only when treated with 30 ng/ml of TNF-α for 24 h. In this study, 20 ng/ml of TNF-α was enough to suppress ApoAI levels by 33% (Figure 4.11).

Pre-treatment with Wedelolactone in the current study abolished the TNF-α-mediated suppression of ApoAI and induction of ApoB gene expression in HepG2 cells (Figure 4.11). However, the mechanism leading to the changes is unknown. Indeed, there is evidence illustrating that the inhibition of NF-κB increases ApoAI and HDL cholesterol through activation of PPARα *in vivo* and *in vitro* (Morishima *et al.*, 2003). Conversely, Xu *et al.* (2006) also showed that PPARα agonists (GW7647) could inhibit ApoB expression in HepG2 cells. In severe primary hypercholesterolaemia, PPARα activator, fenofibrate therapy decreased ApoCIII and lipoprotein particles containing both ApoCIII and ApoB (Bard *et al.*, 1992). Moreover, PPARα agonists were also shown to reduce ApoB levels and increase ApoAI and ApoAII levels (Shah *et al.*, 2010).

Therefore, the effect in the level of both of the gene expression under the impact of TNF-α in this study appears to be mediated by PPARα gene expression. However, there may be other transcription factors that are responsible in the regulation of ApoAI and ApoB genes. Mogilenko *et al.* (2009) demonstrated that treatments of HepG2 cells with chemical inhibitors for JNK, p38 protein kinases, and NF-κB transcription factor abolished the TNF-α-mediated inhibition of human ApoAI gene expression; and other nuclear receptors besides PPARα, such as hepatic nuclear factor 4α (HNF4α) and liver X receptors (LXRs) could directly regulate human ApoAI gene expression in HepG2 cells treated with TNF-α. Moreover, there were also published data obtained from co-transfection experiments in HepG2 cells which revealed that four members of the steroid receptor superfamily, ApoAI

regulatory protein (ARP)-1, v-erbA-related protein (EAR)-2, EAR-3, and HNF4 regulate the expression of ApoB, ApoCIII, and ApoAII genes (Ladias *et al.*, 1992). Taken together, although the molecular mechanisms and the specific transcription factors involved have not yet been defined, available evidences support the view that PPARα could be responsible for the changes in ApoAI and ApoB gene expression during inhibition of NF-κB signalling pathway in HepG2 cells.

5.8   TNF-α represses PPARα expression through canonical NF-κB signalling pathway

Previous experiments have shown that TNF-α-induced NF-κB activation down-regulated PPARα expression (Section 5.5). The NF-κB family is comprised of five closely related members: RelA (p65), c-Rel, RelB, p50 (NF-κB1), and p52 (NF-κB2) (Hayden and Ghosh, 2004). Nevertheless, Tergaonkar (2006) and Bhatnagar *et al.* (2010) established that TNF-α activates both classical (also called canonical) and alternative (also known as non-canonical) NF-κB signalling pathways in skeletal muscle cells. In the classical pathway, NF-κB homo- and heterodimers are composed mainly of RelA (p65), c-Rel, and p50. Conversely, the alternative pathway is composed mainly of RelB and p52 (Demchenko *et al.*, 2010). However, the p50 and p52 proteins are generated by proteolytic processing of their precursors, p105 and p100, respectively (Hayden and Ghosh, 2008). Thus, these findings prompted this study to investigate whether the activation by TNF-α was through the canonical signalling pathway, the non-canonical signalling pathway, or both to down-regulate the PPARα expression.

In resting cells, NF-κB dimers are sequestered inactively in the cytoplasm by IκB proteins. In response to extracellular stimuli, IκB protein is targeted for ubiquitination- and proteasome-dependent degradation when it is specifically phosphorylated by IKK complex. This permits NF-κB to enter the nucleus and activate a number of its target genes (Hayden and Ghosh, 2008; Dhingra *et al.*, 2009). Figure 4.12 shows that the rapid translocation of p50 from the cytoplasmic into the nuclear fraction, and the unchanged p52 expression level in the nuclear fraction. This suggests that classical NF-κB activation may be responsible for the TNF-α suppression of PPARα expression. Conversely, the cleavage of p100 to generate active DNA-binding p52 product is known to occur slower and generally would lead to a delayed activation of nuclear p52-containing complexes (Dejardin, 2006). Unfortunately, there is not enough substantial data in this study to propose that activation of the canonical NF-κB pathway alone may be sufficient to repress PPARα mRNA expression in HepG2 cells. Hence, further confirmation may be necessary.

There were no p105 and p100 detected in the nuclear fractions of HepG2 cells in this experiment. This may be due to the presence of folding ankyrin repeats domain in their C-terminal halves, which is a common feature shared by all IκB family members. Their presence in p105 and p100 would allow them to mask their nuclear localisation signals (NLS), and therefore, p105 and p100 would remain mainly cytosolic (Dejardin, 2006). These findings are consistent with previous data from Souvannavong *et al.* (2007), who also showed that lipopolysaccharide (LPS) triggered the nuclear

translocation of active p50 and p52 in mature B lymphocytes, but p105 and p100 were undetectable in nucleus. Besides, Seubwai *et al.* (2010) also revealed no p100 and p105 were found in the nuclear extracts, but there was a reduction of nuclear translocations of p50, p52, and p65 in a time-dependent manner in cepharanthin-treated cholangiocarcinoma cells. However, work carried out by Derudder and co-researchers (2003) demonstrated that although p105 was undetectable in the nuclear fraction, p100 levels were increased in the nuclear fraction within 4 h after ligation of TNF receptor (TNFR) in mouse embryonic fibroblasts (MEFs). This contradictory result may due to the specificity of different cell types used in the studies.

5.9 TNF-α regulates PPARα via activation of IKK/p105/p50/p65 pathway

5.9.1 IKK-mediated phosphorylation of I*κ*B proteins

Subsequently, the intracellular molecules in the NF-*κ*B signalling pathway were examined by western blot analysis (Section 3.8.4). NF-*κ*B dimers are sequestered in the cytosol of unstimulated cells via non-covalent interactions with a class of inhibitor proteins, called I*κ*Bs. To date, seven I*κ*Bs have been identified: I*κ*Bα, I*κ*Bβ, I*κ*Bγ, I*κ*Bε, Bcl-3, p100, and p105 (Silverman and Maniatis, 2001; Li and Verma, 2002). They contain ankyrin repeats which mediate the association between I*κ*B and NF-*κ*B dimers.

According to Figure 4.13b, d, and f, it was demonstrated that the phosphorylation of IKKα/β (Ser180/181), I*κ*Bα (Ser32), and I*κ*Bβ (Thr19/Ser23) protein levels were significantly increased in the TNF-α-

stimulated HepG2 cells. This result is consistent with existing research which revealed that TNF-α-induced IKK activation is the key step in stimulation of the transcription factor NF-κB (Israël, 2010). Furthermore, the IKK, a trimeric complex composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (also named NF-κB essential modulator or NEMO) mediates the phosphorylation of IκBs on serine residues, and this modification allows their polyubiquitination and destruction by the proteasome (Sun, 2011).

However, the phosphorylated proteins of IκBs are dramatically decreased in the presence of Wedelolactone. Nevertheless, activated IκB proteins were not totally abolished upon Wedelolactone pre-treatment in TNF-α-treated cells. This could be reasoned that since the concentration of Wedelolactone used in this study was low (10 μM), it could only partially inhibit NF-κB activation. Indeed, Maalouf *et al.* (2010) have demonstrated that 60 μM of Wedelolactone almost abrogated the phosphorylation of IκBα protein in epithelial cells; whereas Kobori *et al.* (2004) showed a complete inhibition of phosphorylated IκBα in BALB/c 3T3 cells pre-treated with 100 μM of Wedelolactone. Besides, there may be other pathways which could be involved in activating NF-κB. The molecular events directly upstream of IKK leading to its activation are still not well defined.

Mitogen-activated protein kinase kinase kinase 3 (MEKK3) has been suggested to act upstream of the IKK complex, as it has been shown that cells lacking MEKK3 are partially defective in NF-κB activation in response to certain stimuli (Yang *et al.*, 2001; Huang *et al.*, 2004). Moreover, TNF-α has

been found to cause phosphoinositide 3-kinase (PI3K)/Akt activation (Zhou *et al.*, 2008). It has also been reported that PI3K/Akt induces NF-κB activation by targeting IKKα (Reddy *et al.*, 2000). Activation of Akt has also been shown to increase IκBα degradation and NF-κB activation in macrophages (Sun *et al.*, 2010). Despite this, PI3K/Akt pathway does not induce NF-κB activation in every cell type. Indeed, several studies have shown contradicting observations that the inhibition of PI3K/Akt pathway did not affect TNF-α-induced IKK activation, IκB degradation, and NF-κB-specific DNA protein binding in A549 cells, U937 cells, and hepatocytes (Teshima *et al.*, 2004; Huang and Chen, 2005).

In addition, crosstalk among different signalling has been implicated in many studies. One notable example is a study carried out by Han *et al.* (2010) which revealed that constitutive NF-κB and STAT3 activity are dependent upon one another, and both also depend on heightened PI3K activity in the development of lymphoblastic B-cell lymphomas from mice. Current literature also suggests that at least some of the cytoprotective effects of NF-κB in TNF-α-induced hepatotoxicity are mediated by suppression of JNK activity following TNF-α challenge (Schwabe and Brenner, 2006; Papa *et al.*, 2009). Based on the preliminary results from predecessors (Appendix C), pre-treatment of HepG2 cells with MEK (PD98059), JNK, or PI3K (Wortmannin) inhibitor restored PPARα expression level in TNF-α stimulated cells to basal levels in TNF-α unstimulated cells. However, more studies have to be carried out to elucidate if these pathways do crosstalk to NF-κB signalling in HepG2 cells. Co-treatment with inhibitors against these pathways

could provide useful information regarding the intracellular pathway linkage and signal transduction.

5.9.2 Wedelolactone pre-treatment impairs phosphorylation of p105 at Ser933, but not p100 phosphorylation at Ser866/870

The p105 and p100, which share structural similarity with IκBs in their C-terminal portion, generate the mature p50 and p52 subunits, respectively, through proteosome-mediated processing (Mohamed *et al.*, 2009). Thus, in addition to functioning as IκB-like molecules in liberating specific NF-κB complexes, these proteins also serve as the precursors of p50 and p52. In this study, the degradation or processing of both precursor proteins was constitutive in the absence of TNF-α (Figure 4.14a and d). Although the exact reason remains unknown, it is possible that this process is required for availability of p50 and p52 for maintenance of the different cellular processes supported by NF-κB under basal conditions as suggested by Davis *et al.* (2001). In addition, Silverman and Maniatis (2001) also demonstrated that the processing of p105 is largely constitutive, although certain stimuli may trigger complete degradation or enhanced processing of p105 (Belich *et al.*, 1999; Orian *et al.*, 2000).

Studies have revealed that IKK complex activities can affect NF-κB dimer generation by controlling the processing of precursor proteins (p100 and p105) during or shortly after their translational synthesis (O'Dea and Hoffmann, 2009). Hence, augmentation of the p50 level upon TNF-α stimulation is believed to be mediated by the IKK complex. However, a

sustained level of p52 in TNF-α-stimulated cells may be because to the processing of p100 is tightly regulated (Xiao *et al.*, 2001) and is generally slower (Dejardin, 2006), as mentioned earlier in Section 5.8.

Indeed, IKK mediates phosphorylation of p105 at multiple sites on its carboxy-terminus. A report from Demarchi *et al.* (2003) showed p105 degradation in response to TNF-α was prevented in GSK-3β$^{-/-}$ fibroblasts and by a Ser to Ala point mutation on p105 at positions 903 or 907. The phosphorylation of Ser927, Ser932, and Ser933 by the IKK complex is also essential for TNF-α to trigger p105 ubiquitination and proteolysis (Lang *et al.*, 2003; Satou *et al.*, 2010). Furthermore, numerous reports have also implicated Ser933 is necessary for the p105 stability (Demarchi *et al.*, 2003; Steinbrecher *et al.*, 2005; Siggs *et al.*, 2010). In the current study, the impairment of p105 phosphorylation at Ser933 in the presence of Wedelolactone (Figure 4.14b) correlates with the review article by Hayden and Ghosh (2008) who discussed that p105 is phosphorylated by IKK, targeting it for degradation to release associated NF-κB subunits.

Interestingly, there were no changes on the phosphorylation of p100 at Ser866 and Ser870 in Wedelolactone pre-treated HepG2 cells (Figure 4.14e). There were findings which suggested that even though the two amino acid residues, Ser866 and Ser870 of p100, are required for the recruitment of IKKα to p100, but they are not the actual phosphorylation sites for IKKα (Qu *et al.*, 2004; Xiao *et al.*, 2004). Xiao *et al.* (2004) and Shih *et al.* (2011) revealed that after IKK was recruited to p100 in a Ser866 and Ser870-

dependent manner, IKKα has been shown to phosphorylate p100 at Ser99, Ser108, Ser115, Ser123, and Ser872, which provide a signal for p100 to be recognised by the SCF/βTrCP ubiquitin ligase complex. On the other hand, Sun (2011) demonstrated that binding of beta-transducing repeat-containing protein (βTrCP) to p100 is dependent on these two serine phosphorylation residues. Mutation of one or both of these serines completely abolished the inducible processing of p100 (Xiao *et al.*, 2001; Liang *et al.*, 2006). However, this study showed that Ser866 and Ser870 of p100 are not involved in mediating the TNF-α repression on PPARα expression. Here, the phosphorylation of p105 at Ser933, but not p100 at Ser866/870, seem to play an important role in the TNF-α response instead.

5.9.3 Wedelolactone pre-treatment abrogated p65 phosphorylation at Ser468 and Ser536 in the cytoplamic fraction

The activity of NF-κB is controlled at several levels including the phosphorylation of the strongly transactivating p65 (RelA) subunit. Geisler *et al.* (2007) has proposed that p65 is essential for TNF-α-induced NF-κB activation in adult hepatocytes, and several reports have lent support to the proposal that p65 (RelA) phosphorylation is required for efficient transcriptional activation (Wang *et al.*, 2000; Madrid *et al.*, 2001). Thus far, ten different phosphorylation sites have been mapped for activating p65 subunit activation. Five sites (Thr254, Ser276, Ser205, Ser281, and Ser311) are contained in the N-terminal Rel homology domain (RHD), whereas five sites (Ser468, Thr435, Thr505, Ser529, and Ser536) are contained within C-terminal transactivation domain (TAD) (Anrather *et al.*, 2005; Mattioli *et al.*,

2006; Neumann and Naumann, 2007; Xing *et al.*, 2011). Although the phosphorylation of p65 is found at many sites, the most extensively studied are Ser276, Ser468, and Ser536 (Cullen, 2009; Moreno *et al.*, 2010). These three specific serine residues have contributed to NF-κB transactivation activity (Mattioli *et al.*, 2006).

This current study has identified that there is an inducible phosphorylation of Ser276, Ser468, and Ser536 in the cytoplasm in response to TNF-α-stimulation (Figure 4.15b – d). This result is in line with the study by Seidel *et al.* (2009) who reported a similar induction in phosphorylation by TNF-α in airway smooth muscle cells. Inducible phosphorylation of Ser276 and Thr311 promotes the interaction of p65 with the coactivating acetylase CREB-binding protein/p300, thus leading to p65 acetylation (Lys310) and NF-κB-driven transcription (Mattioli *et al.*, 2006; Neumann and Naumann, 2007; Reber *et al.*, 2009). Besides, phosphorylated p65 at Ser276 could interact with RelB in the nucleus (Jacque *et al.*, 2005). However, these p65/RelB heterodimers are transcriptionally inactive complexes which do not bind to NF-κB sites on the promoter and subsequently inhibit RelB-mediated NF-κB activity. Phosphorylation of Ser276 may also be caused by other factors, such as the mitogen- and stress-activated protein kinase-1 (MSK1) (Anest *et al.*, 2004) and protein kinase A catalytic subuit (PKAc) (Jamaluddin *et al.*, 2007), which phosphorylate p65 at Ser276 independently of IκBα degradation (Reber *et al.*, 2009). Nevertheless, Zhong *et al.* (1997) showed a contradictory result which demonstrated that PKA phosphorylates p65 at Ser276 during IκB degradation in rabbit lung cells. Hence, such mechanisms could explain at

least in part, the inhibition of IKK activity by Wedelolactone has no effect on Ser276 levels in Wedelolactone pre-treated cells when compared to the Wedelolactone untreated cells in this study (Figure 4.15b).

Mattioli and co-reseachers (2006) have demonstrated that phosphorylation of Ser468 and Ser536 in mouse embryo fibroblasts (MEFs) enhances the transcriptional activity of p65 by using transient reporter gene assays. However, a contradictory finding appears to be Ser468 diminishes NF-κB-dependent transcription after TNF-α and IL-1β stimulation in HeLa cells (Schwabe and Sakurai, 2005). From this study, pre-treatment of cells with Wedelolactone effectively blocked the cytoplasmic phosphorylation of p65 at Ser468 and Ser536, but not Ser276. Since IKK has been reported as an upstream kinase which phosphorylates p65 (RelA) (Yang *et al.*, 2003), it can be postulated that the phosphorylation at these two serine residues (468 and 536) are dependent on IKK activation in HepG2 cells and are crucial in mediating the TNF-α action on PPARα gene expression. This is consistent with the findings of others that IKK is known to phosphorylate p65 at Ser468 in T cell, B cell, cervix carcinoma, hepatoma, breast cancer, and primary hepatic stellate cells (Schwabe and Sakurai, 2005; Mattioli *et al.*, 2006); and at Ser536 in T cell, HeLa cells, breast cancer, and embryonic kidney cells (Buss *et al.*, 2004; O'Mahony *et al.*, 2004; Adli and Baldwin, 2006). In contrast, Sasaki *et al.* (2005b) have assigned an IκB-independent p65 phosphorylation on Ser536, in regulating the NF-κB pathway. Thus, it can be deduced that distinct mechanisms in the signalling cascade may be involved in different p65 phosphorylation sites, depending on the stimulus or cell type studied. In this

study, phosphorylation of p65 at Ser468 and Ser536 seem to play important roles in mediating TNF-α inhibitory effect on PPARα in HepG2 cells.

5.9.4   Involvement of phosphorylation of p65 at Ser468 and Ser536 in the nuclear fraction

The p65 is predominantly localised in the cytoplasm (O'Mahony *et al.*, 2004). Upon TNF-α-stimulation, there was no change in the level of cytoplasmic p65 (Figure 4.15a), but an increase was observed in the nuclear fractions of p65 (Figure 4.16a) which implicated that TNF-α induced the nuclear translocation of p65 in HepG2 cells. The observation of p65 nuclear translocation in unstimulated cells is consistent with the review by Oeckinghaus and Ghosh (2009) who stated that p65 could also be constitutively detectable in nuclear fractions in certain cell types, such as B cells, macrophages, neurons, and vascular smooth muscle cells, as well as a large number of tumour cells. The p65 nuclear level, however, was reduced in Wedelolactone pre-treated cells. This suggests that although the signalling was somewhat impaired, the release of p65 was intact but inhibited. To support this, similar findings by Jeong *et al.* (2005) also revealed that the nuclear p65 was not totally abrogated in IKKα$^{-/-}$ MEF cells. However, the exact reason remains unknown.

Nonetheless, although liberation of p65 by TNF-α treatment to nucleus, which is essential for its activation, emerging evidence has indicated that it is not sufficient to activate NF-κB-dependent transcription (Sizemore *et al.*, 2002; Zhong *et al.*, 2002). Several lines of evidence have suggested that

the post-translational modification state such as phosphorylation or acetylation events of p65 lead to an increase in p65 DNA binding and/or transcriptional activity, though the mechanisms are not completely understood (Okazaki *et al.*, 2003; Nowak *et al.*, 2008). It is likely that these modification sites would serve to fine-tune NF-κB transcriptional activity, which is to determine target-gene specificity and timing of gene expression, rather than acting as simple on-off switches. Based on Figure 4.16b – d, phosphorylation sites of p65 at Ser276, Ser468, and Ser536 was also detectable in the nuclear fractions of TNF-α stimulated cells. However, phosphorylation of p65 at Ser276 and Ser468 was more prominent than at Ser536 in the nucleus judging from the intensity of the phosphorylations in this study. Indeed, similar studies have revealed that phosphorylation at Ser276 and Ser468 was predominant in the nucleus and phosphorylated p65 at Ser536 remained mainly localised in cytoplasm (Mattioli *et al.*, 2006; Nowak *et al.*, 2008; Moreno *et al.*, 2010). However, the possibility of phosphorylated Ser536 of p65 detected mostly in cytoplasm may be due to the rapid dephosphorylation in the nucleus in certain cell types by certain protein phosphatases (Sakurai *et al.*, 2003).

Suprisingly, only a slight impairment in the level of Ser276 phosphorylated nuclear p65 protein upon pre-treatment with Wedelolactone was seen (Figure 4.16b), thus raising the possibility that this phosphorylation could be activated through an IκB-independent signalling pathway. Since phosphorylated p65 at Ser276, but not Ser468 or Ser536 was observed in Wedelolactone treated cells, it could be concluded that Ser276 was not involved in TNF-α repression of PPARα.

Studies have also shown that the impact of p65 phosphorylation at Ser468 and Ser536 on gene expression are highly specific and occur at distinct intracellular locations. For example, chromatin immunoprecipitation (ChIP) analysis using antibody specific against the Ser536-phosphorylated form of p65 proved that the Ser536 is specifically recruited to the IL-8 promoter, but not to TNF-α, IL-1β, or IL-6 promoters, to regulate the ability of nuclear IκBα in inhibiting the subsequent NF-κB binding to IL-8 promoter in human leukocytes (Ghosh *et al.*, 2010). This forms the basis of potential autoregulatory negative feedback loop that allows transient activation of NF-κB (Hay *et al.*, 1999). Phosphorylation of p65 at Ser468 has been shown to be both stimulatory and inhibitory to NF-κB, depending on the cellular signal and kinase involved (Mattioli *et al.*, 2006; Wittwer and Schmitz, 2008; Geng *et al.*, 2009). Moreover, reporter gene assays showed that prevention of Ser468 or Ser536 phosphorylation stimulates *Icam1*, *Vcam1*, and *Csf2* expression. In contrast, mutation of either these sites impairs *Saa3*, *Mmp3*, and *Mmp13* transcription (Moreno *et al.*, 2010). These transcripts are known to be linked particularly to inflammation and/or lipid metabolism (Leinonen *et al.*, 2003; Cvoro *et al.*, 2008; Rubin *et al.*, 2008; O'Hara *et al.*, 2012). In view of the above findings, it is indicated that TNF-α-mediated NF-κB-dependent target genes play a critical role in regulating the PPARα expression.

Considering the cross-talk between PPARα and NF-κB, most emphasis has been generally focusing on the effects of PPARα or PPARα ligands on NF-κB. In this present study, it is suggested that the reverse situation, namely p65-dependent inhibition of PPARα-mediated

transactivation is an important facet in the regulation of PPARα-dependent process. Indeed, there are some evidences which have indicated p65 as a potent repressor of PPAR-mediated transactivation. It was reported by Westergaard and co-researchers (2003) that p65-dependent repression of PPARδ-mediated transactivation was partially relieved by forced expression of the coactivators p300 and CBP. Overexpression of p65 was also shown to inhibit induction of a PPARγ-responsive reporter gene by activated PPARγ in a dose-dependent manner (Ruan *et al.*, 2003). Of relevance to this study is the fact that p65 phosphorylation at Ser468 and Ser536 are most probably IKK-dependent and this is the first reported instance that these phosphorylations are crucial in mediating the actions of TNF-α on PPARα gene in HepG2 cells. Furthermore, Figure 5.1 has showed the overall hypothetical working mechanism in this study.

5.10   Hypothesis on the NF-κB dimers involved in TNF-α down-regulation of PPARα

NF-κB refers to a hetero- or homodimer composed of two of the five Rel family member including p65 (RelA), RelB, c-Rel, NF-κB1/p50 (precursor p105), and NF-κB2/p52 (precursor p100) (Hayden and Ghosh, 2004; Hayden and Ghosh, 2008). Different dimers are held in the cytoplasm by interaction with specific inhibitors. It has been known that each NF-κB dimer is able to selectively regulate a few target promoters; however, several genes are redundantly induced by more than one dimer (Saccani *et al.*, 2003). Whether this property simply generates redundancy in target gene activation or underlies more complex regulatory mechanisms is an open issue. In

111
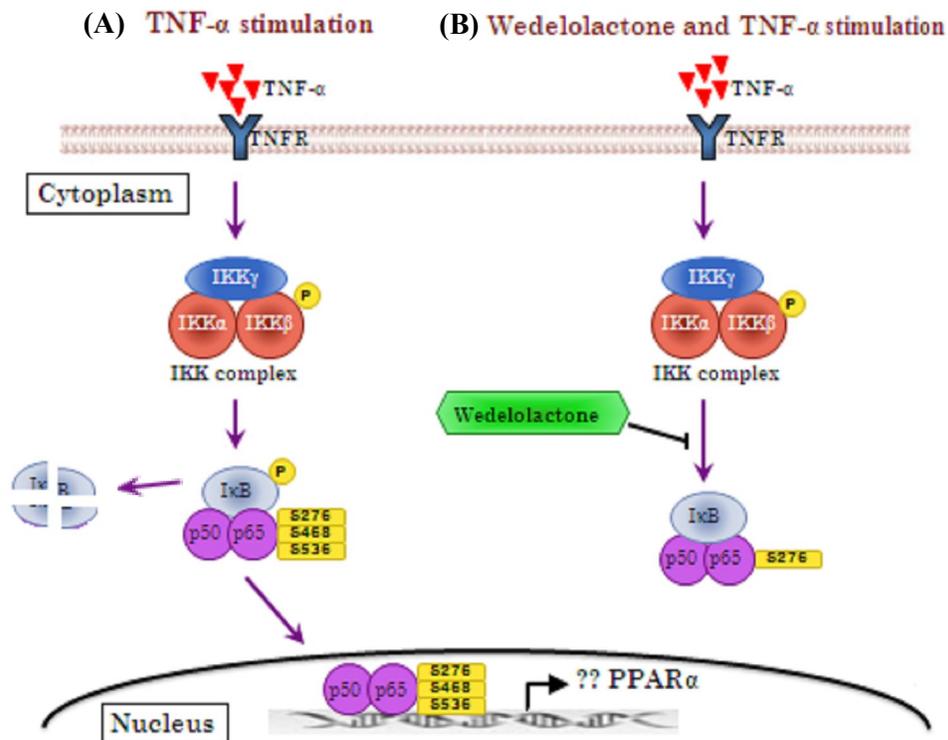
Figure 5.1: A schematic depiction of the hypothetical mechanism of action of TNF-α on PPARα suppression in HepG2 cells.

(A) Upon TNF-α stimulation, ligation of TNF receptor (TNFR) with their ligands leads to initiation of NF-κB signalling pathway. An inhibitory protein, IκB that normally binds to NF-κB and inhibits its translocation is phosphorylated by activated IKK and subsequently degraded, releasing NF-κB. Subsequently, phosphorylation of p65 has been found at Ser276, Ser468, and Ser536. The NF-κB dimer translocates to the nucleus and mediates the relevant gene transcription. However, the link to the PPARα expression remains elusive. (B) In the present of Wedelolactone, activated IKK fails to phosphorylate the inhibitory protein. Surprisingly, there is no effect on the phosphorylated p65 at Ser276. However, phosphorylation of p65 at Ser468 and Ser536 are severely abrogated. Arrows represent activation mechanisms; 'T' bar indicates inhibition; 'P' circles indicate phosphorylation events.

addition, *in vitro* studies have suggested that homo- or heterodimers can positively or negatively affect transactivation of NF-κB-controlled genes (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992; Fujita *et al.*, 1992; Grilli *et al.*, 1993). As shown in earlier experiments (Section 5.8), TNF-α down-regulates PPARα most likely through p105 and p50, whereas TNF-α-induced transactivation function of p65 at Ser468 and Ser536 are important for the NF-κB-dependent down-regulation of TNF-α on PPARα. Thus, the data here have demonstrated the possibility that the importance of p50 and p65 in the TNF-α inhibition of PPARα expression.

Theoretically, the transcriptional activation domain (TAD) necessary for the positive regulation of gene expression is present only in p65, c-Rel, and RelB (Hayden and Ghosh, 2008). As they lack TADs, p50 and p52 may repress transcription unless they are associated with a TAD-containing NF-κB family member or other proteins capable of coactivator recruitment. Consequently, p50 homodimers which retain their ability to bind to NF-κB sites are thought to be transcriptional repressors. For instance, p50 homodimer has been shown to reduce the enhancer effect of NF-κB site and this results in a reduction of LPS-inducible gene expression in primary human monocytes (Udalova *et al.*, 2000). Satou *et al.* (2010) also revealed that homodimer formation of p50/p50 could also play a role in mediating TNF-α repression of genes.

However, *in vitro* studies have shown that p50 can associate with other transcriptional activators, such as Bcl-3 (Fujita *et al.*, 1993) or p300

(Deng and Wu, 2003), to activate transcription. A study carried out by Cao *et al.* (2006) also has demonstrated that p50 homodimers can be transcriptional activators of IL-10 in macrophages. Not only p50 homodimer, p65 homodimer was also shown to stimulate NF-κB-directed transcription. Comparative functional studies with transfected genes have suggested that p65 homodimers can induce NF-κB-dependent gene expression in the absence of p50 (Schmitz and Baeuerle, 1991; Ballard *et al.*, 1992). Likewise, the NF-κB site taken from the IL-8 promoter was shown to be optimally bound and activated by the p65 homodimers and not by the p50/p65 heterodimers (Kunsch and Rosen, 1993). Furthermore, Marui *et al.* (2005) reported that TNF-α induced p65 homodimers may actively participate in TNF-α regulation of NF-κB-dependent gene expression in endothelial cells.

The p50/p65 heterodimer is the most ubiquitous, constitutive, and inducible member of the Rel family of transcription factors (Chen *et al.*, 1998). The heterodimer has been extensively linked to the orchestration of diverse cellular functions such as cell growth, development, inflammatory responses and coordination of the expression of multiple inflammatory and innate immune genes (Chen *et al.*, 1998; Pahl, 1999). Inactivation of p65 and p50 individually was shown to result in early degeneration of liver tissue; while in p50/p65 double knockout mice were embryonically lethal mainly due to extensive liver apoptosis (Beg *et al.*, 1995; Horwitz *et al.*, 1997). Liu and co-reseachers (2000) showed the ability of p50/p65 heterodimer in activating the human TNF-α promoter through the NF-κB binding site in LPS-treated macrophages. Moreover, other studies also concluded the essential role of the

p50/p65 heterodimer in transcriptional regulation of tissue factor in activated vascular endothelial cells and stimulated monocytes/macrophages (Li *et al.*, 2009). Here, it is postulated that IKK/p50/p65 is the likely regulator for the down-regulation of PPARα and PPARα regulated gene expression. Unfortunately, there is no concrete evidence for the specific dimers that took place in PPARα repression in this present study. Therefore, the precise molecular mechanism of protein-promoter interactions needs to be further studied.

5.11   Future studies

The complexity of the network of molecular mechanism is owed to the heterogenicity, cell specificity, and pleiotropy of their effects. Hence, results obtained in the present study were specifically for HepG2 cells under particular experimental conditions. PPARα is first and foremost transcription factor that executes intricate regulation of gene networks, but is in turn subjected to complex fine-tuning and control itself. It is regulated by and intersects with diverse signalling pathways through positive or negative feedback mechanisms. For example, TNF-α has been shown to increase mammalian target of rapamycin (mTOR) phosphorylation (Tsou *et al.*, 2010; Ng *et al.*, 2011). In a study by Parent *et al.* (2007), mTOR activation impaired the transcription of the transcription factors PPARα, PPARδ, and RXRβ and their target genes. Inevitably, thorough studies in future would help produce better understanding of these molecular underpinnings.

Since NF-κB has been shown to have an impact in the effect of TNF-α on reduction of PPARα expression, additional work such as electrophoretic mobility shift assay (EMSA), supershift assay, and gene silencing of NF-κB subunits could be performed to ascertain the specificity and subunit composition of NF-κB involved in PPARα repression by TNF-α. Since TNF-α also affects the intracellular localisation of the kinases, immunofluorescence studies could be carried out to better define the localisation of endogenous kinases in TNF-α-stimulated cells for various periods.

As inhibition of NF-κB pathway have been implicated in the abrogation of TNF-α down-regulation of PPARα in this study, it is imperative to investigate the effects of NF-κB inhibitors on the regulatory potential of PPARα. To understand better the mechanism that is operative in the increase in PPARα expression, PPARα promoter activity before and after TNF-α stimulation in the absence or presence of Wedelolactone could be examined with a functional promoter reporter assay through transient transfection with luciferase reporter gene. Furthermore, the impact of each individual p65 phosphorylation site is highly gene specific. At the present time, concrete evidence supporting the mechanism behind TNF-α-mediated down-regulation of PPARα through phosphorylation of p65 at serine 468 and 536 remains elusive. However, these gene regulations are not only confined to phosphorylation sites, but also through p65 modifications by acetylation or monomethylation as reported by Buerki *et al.* (2008), Ea and Baltimore (2009), and Rothgiesser *et al.* (2010). Therefore, it is also imperative to identify whether the NF-κB post-translational modifications alone or in combination

could generate distinct patterns that function to direct transcription in a target gene-specific fashion.

Missing links also exist if the NF-κBs participate directly or indirectly in the control of PPARα gene expression. Systematic approaches such as ChIP assay or combined with genomic tiling microarray (ChIP on chip) experiments could help address these missing gaps in the future. Also, it is currently unclear whether the intracellular TNF-α signalling pathway regulating PPARα has different specificities under *in vitro* and *in vivo* conditions. The study could be extended to *in vivo* experiments by generating PPARα or NF-κB knockout mice and using the small interfering RNA (siRNA)- or short hairpin RNA (shRNA)-mediated gene silencing to understand the functions and underlying mechanisms. These experiments should further our understanding of the complexities of the signalling mechanisms of TNF-α pathway.

CHAPTER 6

CONCLUSION

In this study, PPARα mRNA and protein expression were shown to be down-regulated by TNF-α in a dose- and time-dependent manner. This down-regulation was proven to be mediated by the NF-κB pathway primarily at the rate of PPARα gene transcription. The pre-treatment of NF-κB activation inhibitors, such as Wedelolactone, SC-514, and NF-κB activation inhibitor IV prior to TNF-α treatment restored the PPARα expression levels.

Subsequent experiments also demonstrated that Wedelolactone pre-treatment were associated with changes on PPARα-regulated genes. Wedelolactone pre-treatment caused an increased and decreased in apolipoprotein AI and B gene expression after TNF-α treatment, respectively. This further suggests that TNF-α suppression of the PPARα activity and its target genes may be, at least in part mediated through NF-κB activation.

Furthermore, western blot analyses indicated that phosphorylation of IκBα at Ser32, IκBβ at Thr19/Ser23, and p105 at Ser933 were shown to be mediated by IKK activity. In contrast, phosphorylation of p100 at Ser866/870 remained activated in the presence of Wedelolactone, indicating the activation of p100 is IKK-independent. Meanwhile, TNF-α stimulation alone resulted in a marked increase in the level of nuclear p50 protein, but expression of nuclear p52 protein was not affected. This suggests that TNF-α represses PPARα

expression through the canonical NF-κB signalling pathway. Phosphorylation of p65 at Ser276, Ser468, and Ser536 were shown to be induced and translocated into the nucleus after TNF-α treatment. However, the presence of Wedelolactone abolished the p65 phosphorylation at Ser468 and Ser536, but it did not influence the phosphorylation of Ser276. Overall, the results demonstrated that the reduction of PPARα gene expression in HepG2 cells is signalled through the IKK/p105/p50/p65 pathway.

In conclusion, this study has successfully elucidated the upstream signalling components mediating PPARα down-regulation by TNF-α, and identified for the first time that specific p65 phosphorylation sites, Ser468 and Ser536 are involved. Nevertheless, interconnected signalling cascades between NF-κB and PPARα are complex. Further investigation to support the mechanism and the involvement of exact NF-κB dimerisation would be necessary in future studies.

REFERENCES

't Hoen, P. A., Hirsch, M., de Meijer, E. J., de Menezes, R. X., van Ommen, G. B., den Dunnen, J. T. (2011). mRNA degradation controls differentiation state-dependent differences in transcript and splice variant abundance. *Nucl Acids Res*, 39(2), 556–566.

Adkins, J. N., Varnum, S. M., Auberry, K. J., Moore, R. J., Angell, N. H., Smith, R. D., Springer, D. L., Pounds, J. G. (2002). Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. *Mol Cell Proteomics*, 1, 947–955.

Adli, M., Baldwin, A. S. (2006). IKK-i/IKKε controls constitutive, cancer cell-associated NF-κB activity via regulation of Ser-536 p65/RelA phosphorylation. *J Biol Chem*, 281, 26976–26984.

Aggarwal, B. B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol*, 3, 745–756.

Aggarwal, B. B., Takada, Y. (2005). Pro-apoptotic and anti-apoptotic effects of TNF in tumour cells. Role of nuclear transcription factor NF-κB. *Cancer Treat Res*, 126, 103–127.

Aldridge, G. M., Podrebarac, D. M., Greenough, W. T., Weiler, I. J. (2008). The use of total protein stains as loading controls: an alternative to high-abundance single protein controls in semi-quantitative immunblotting. *J Neurosci Methods*, 172(2), 250–254.

Anest, V., Cogswell, P. C., Baldwin, A. S. Jr. (2004). IκB kinase α and p65/RelA contribute to optimal epidermal growth factor-induced c-fos gene expression independent of IκBα degradation. *J Biol Chem,* 279, 31183–31189.

Anrather, J., Racchumi, G., Iadecola, C. (2005). *cis*-acting element-specific transcriptional activity of differentially phosphorylated NF-κB. *J Biol Chem*, 280, 244–252.

Ansquer, J. C., Foucher, C., Rattier, S., Taskinen, M. R., Steiner, G. (2005). Fenofibrate reduces progression to microalbuminuira over 3 years in a placebo-controlled study in type 2 diabetes: Results from the Diabetes Atherosclerosis Intervention Study (DAIS). *Am J Kidney Dis*, 45(3), 485–493.

Auboeuf, D., Rieusset, J., Fajas, L., Vallier, P., Frering, V., Riou, J. P., Steals, B., Auwerx, J., Laville, M., Vidal, H. (1997). Tissue distribution and quantification of the expression of mRNAs of PPARs and liver X receptor-alpha in humans: No alteration in adipose tissue of obese and NIDDM patients. *Diabetes*, 46(8), 1319–1327.

Auburn, R. (2004). *RNA quality control.* URL: http://www.flychip.org.uk/protocols/gene_expression/rna_qc.pdf. Accessed on 26th January 2011.

Baeuerle, P. A. (1991). The inducible transcription activator NF-κB: Regulation by distinct protein subunits. *Biochim Biophys Acta*, 1072(1), 63–80.

Balkwill, F. (2009). Tumour necrosis factor and cancer. *Nat Rev Cancer*, 9, 361–371.

Ballard, D. W., Dixon, E. P., Peffer, N. J., Bogerd, H., Doerre, S., Stein, B., Greene, W. C. (1992). The 65-kDa subunit of human NF-κB functions as a potent transcriptional activator and a target for v-Rel-mediated repression. *Proc Natl Acad Sci USA*, 89, 1875–1879.

Bard, J. M., Parra, H. J., Camare, R., Luc, G., Ziegler, O., Dachet, C., Bruckert, E., Douste-Blazy, P., Drouin, P., Jacotot, B., De Gennes, J. L., Keller, U., Fruchart, J. C. (1992). A multicenter comparison of the effects of simvastatin and fenofibrate therapy in severe primary hypercholesterolemia, with particular emphasis on lipoproteins defined by their apolipoprotein composition. *Metabolism*, 41(5), 498–503.

Bardot, O., Aldridge, T. C., Latruffe, N., Green, S. (1993). PPAR-RXR heterodimer activates a peroxisome proliferators response element upstream of the bifunctional enzyme gene. *Biochem Biophys Res Commun*, 192, 37–45.

Barger, P. M., Brandt, J. M., Leone, T. C., Weinheimer, C. J., Kelly, D. P. (2000). Deactivation of PPARα during cardiac hypertrophic growth. *J Clin Invest*, 105(12), 1723–1730.

Barger, P. M., Browning, A. C., Garner, A. N., Kelly, D. P. (2001). p38 mitogen-activated protein kinase activates PPARα: a potential role in the cardiac metabolic stress response. *J Biol Chem*, 276, 44495–44501.

Bartolomé, N., Rodríguez, L., Martínez, M. J., Ochoa, B., Chico, Y. (2007). Upregulation of apolipoprotein B secretion, but not lipid, by TNF-α in rat hepatocyte cultures in the absence of extracellular fatty acids. *Ann NY Acad Sci*, 1096, 55–69.

Bas, A., Forsberg, G., Hammarström, S., Hammarström, M. L. (2004). Utility of the housekeeping genes 18S rRNA, β-actin, and GAPDH for normalisation in Real-Time quantitative reverse transcriptiase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand J Immunol*, 59(6), 566–573.

Becker, J., Delayre-Orthez, C., Frossard, N., Pons, F. (2008). Regulation of PPARα expression during lung inflammation. *Pulm Pharmacol Ther*, 21(2), 324–330.

Beg, A. A., Sha, W. C., Bronson, R. T., Gosch, S., Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. *Nature*, 376, 167–170.

Behar, M., Hao, N., Dohlman, H. G., Elston, T. C. (2007). Mathematical and computational analysis of adaption via feedback inhibition in signal transduction pathways. *Biophys J*, 93, 806–821.

Belich, M. P., Salmerón, A., Johnston, L. H., Ley, S. C. (1999). TPL-2 kinase regulates the proteolysis of the NF-κB-inhibitory protein NF-κB1 p105. *Nature*, 397(6717), 363–368.

Berasain, C., Castillo, J., Perugorria, M. J., Latasa, M. U., Prieto, J., Avila, M. A. (2009). Inflammation and liver cancer: New molecular links. *Ann NY Acad Sci*, 1155, 206–221.

Berger, J. P., Akiyama, T. E., and Meinke, P. T. (2005). PPARs: therapeutic targets for metabolic disease. *Trends Pharmacol Sci*, 26, 244–251.

Berger, J., Moller, D. E. (2002). The mechanisms of action of PPARs. *Annu Rev Med*, 53, 409–435.

Berman, K. S., Verma, U. N., Harburg, G., Minna, J. D., Cobb, M. H., Gaynor, R. B. (2002). Sulindac enhances TNF-α-mediated apoptosis of lung cancer cell lines by inhibition of NF-κB. *Clin Cancer Res*, 8, 354.

Bhatnagar, S., Panguluri, S. K., Gupta, S. K., Dahiya, S., Lundy, R. F., Kumar, A. (2010). TNF-α regulates distinct molecular pathways and gene networks in cultured skeletal muscle cells. *PLoS One*, 5(10), e13262.

Bjarnadottir, H., Jonsson, J. J. (2005). A rapid Real-Time qRT-PCR assay for ovine beta-actin mRNA. *J Biotechnol*, 117(2), 173–182.

Blanquart, C., Mansouri, R., Paumelle, R., Fruchart, J. C., Staels, B., Glineur, C. (2004). The PKC signaling pathway regulates a molecular switch between transactivation and transrepression activity of the PPARα. *Mol Endocrinol*, 18, 1906–1918.

Blattner, C., Sparks, A., Lane, D. (1999). Transcription factor E2F-1 is upregualted in response to DNA damage in a manner analogous to that of p53. *Mol Cell Biol*, 19(5), 3704–3713.

Bonizzi, G., Bebien, M., Otero, D. C., Johnson-Vroom, K. E., Cao, Y., Vu, D., Jegga, A. G., Aronow, B. J., Ghosh, G., Rickert, R. C., Karin, M. (2004). Activation of IKKα target genes depends on recognition of specific κB binding sites by RelB:p52 dimers. *EMBO J*, 23, 4202–4210.

Bradford, R. H., Goldberg, A. C., Schonfeld, G., Knopp, R. H. (1992). Double-blind comparison of bezafibrate versus placebo in male volunteers with hyperlipoproteinemia. *Atherosclerosis*, 92(1), 31–40.

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPARα, β, and γ in the adult rat. *Endocrinol*, 137, 354–366.

Brown, J. D., Oligino, E., Rader, D. J., Saghatelian, A., Plutzky, J. (2011). VLDL hydrolysis by hepatic lipase regulates PPARδ transcriptional responses. *PLos ONE*, 6(7), e21209.

Brown, P. J., Smith-Oliver, T. A., Charifson, P. S., Tomkinson, N. C., Fivush, A. M., Sternbach, D. D., Wade, L. E., Orband-Miller, L., Parks, D. J., Blanchard, S. G., Kliewer, S. A., Lehmann, J. M., Willson, T. M. (1997). Identification of peroxisome proliferator-activated receptor ligands from a biased chemical library. *Chem Biol*, 4(12), 909–918.

Brown, P. J., Stuart, L. W., Hurley, K. P, Lewis, M. C., Winegar, D. A., Wilson, J. G., Wilkison, W. O., Ittoop, O. R., Willson, T. M. (2001). Identification of a subtype selective human PPARα agonist through parallel-array synthesis. *Bioorg Med Chem Lett*, 11(9), 1225–1227.

Brown, P. J., Winegar, D. A., Plunket, K. D., Moore, L. B., Lewis, M. C., Wilson, J. G., Sunseth, S. S., Koble, C. S., Wu, Z., Chapman, J. M., Lehmann, J. M., Kliewer, S. A., Wilson, T. M. (1999). A ureido-thioisobutyric acid (GW9578) is a subtype-selective PPARα agonist with potent lipid-lowering activity. *J Med Chem*, 42(19), 3785–3788.

Buerki, C., Rothgiesser, K. M., Valovka, T., Owen, H. R., Rehrauer, H., Fey, M., Lane, W. S., Hottiger, M. O. (2008). Functional relevance of novel p300-mediated lysine 314 and 315 acetylation of RelA/p65. *Nucl Acids Res*, 36(5), 1665–1680.

Bumrungpert, A., Kalpravidh, R. W., Chitchumroonchokchai, C., Chuang, C. C., West, T., Kennedy, A., McIntosh, M. (2009). Xanthones from mangosteen prevent lipopolysaccharide-mediated inflammation and insulin resistance in primary cultures of human adipocytes. *J Nutr*, 139, 1185–1191.

Burns, K. A. (2007). *Regulation of peroxisome proliferator-activated receptor alpha activity via cross-talk with glycogen synthase kinase* 3. Ph. D. Thesis, The Pennsylvania State University, United States.

Buss, H., Dorrie, A., Schmitz, M. L., Hoffmann, E., Resch, K., Kracht, M. (2004). Constitutive and IL-1-inducible phosphorylation of p65 NF-κB at serine 536 is mediated by multiple protein kinases including IκB kinases IKKα, IKKβ, IKKε, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA binding protein-associated factor II31-mediated IL-8 transcription. *J Biol Chem*, 279, 55633–55643.

Cabrero, A., Alegret, M., Sanchez, R. M., Adzet, T., Laguna, J. C., Carrera, M. V. (2002). Increased reactive oxygen species production down-regulates PPARα pathway in C2C12 skeletal muscle cells. *J Biol Chem*, 277(12), 10100–10107.

Calkin, A. C., Cooper, M. E., Jandeleit-Dahm, K. A. (2006). Gemfibrozil decreases atherosclerosis in experimental diabetes in association with a reduction in oxidative stress and inflammation. *Diabetologia*, 49(4), 766–774.

Cao, S., Zhang, X., Edwards, J. P., Mosser, D. M. (2006). NF-*κ*B1 (p50) homodimers differentially regulate pro- and anti-inflmmatory cytokines in macrophages. *J Biol Chem*, 281(36), 26041–26050.

Castillo, G., Brun, R. P., Rosenfield, J. K., Hauser, S., Park, C. W., Troy, A. E., Wright, M. E., Spiegelman, B. M. (1999). An adipogenic cofactor bound by the differentiation domain of PPARγ. *EMBO J*, 18, 3676−3687.

Cawthorn, W. P., Sethi, J. K. (2008). TNF-α and adipocyte biology. *FEBS Lett*, 582(1), 117–131.

Chacko, S. (2005). *High quality RNA: The foundation for successful microarray experiments*. URL: http://www.biotech-online.com/fileadmin/pdf/datasheet/high-quality-rna-the-foundation-for-successful-microarray-experiments.pdf. Accessed on 10th February 2012.

Chait, A., Brazg, R. L., Tribble, D. L. (1993). Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. *Am J Med*, 94(4), 350–356.

Chakrabarti, R., Vikramadithyan, R. K., Misra, P., Hiriyan, J., Raichur, S., Damarla, R., Gershome, C., Suresh, J., Rajagopalan, R. (2003). Ragaglitazar: a novel PPARα and PPARγ agonist with potent lipid-lowering and insulin-sensitising efficacy in animal models. *Br J Pharmacol*, 140(3), 527–537.

Chakravarthy, M. V., Zhu, Y., López, M., Yin, L., Wozniak, D. F., Coleman, T., Hu, Z., Wolfgang, M., Vidal-Puig, A., Lane, M. D., Semenkovich, C. F. (2007). Brain fatty acid synthase activates PPARα to maintain energy homeostasis. *J Clin Invest*, 117(9), 2539–2552.

Chan, M. M., Evans, K. W., Moore, A. R., Fong, D. (2010). PPAR: Balance for survival in parasitic infections. *J Biomed Biotechnol*, 2010, 828951.

Chang, F., Jaber, L. A., Berlie, H. D., O'Connell, M. B. (2007). Evolution of peroxisome proliferator-activated receptor agonists. *Ann Pharmacother*, 41(6), 973–983.

Chawla, A., Lee, C., Barak, Y., He, W., Rosenfeld, J., Liao, D., Han, J., Kang, H., Evans, R. (2003). PPARδ is a very low-density lipoprotein sensor in macrophages. *Proc Natl Acad Sci USA*, 100, 1268–1273.

Chen, F. E., Huang, D. B., Chen, Y. Q., Ghosh, G. (1998). Crystal structure of p50/p65 heterodimer of transcription factor NF-κB bound to DNA. *Nature*, 391(6665), 410–413.

Chen, J. D., Evans, R. M., (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*, 377, 454–457.

Chen, L. F., Greene, W. C. (2004). Shaping the nuclear action of NF-κB. *Nat Rev Mol Cell Biol*, 5, 392–401.

Chen, X., Thibeault, S. L. (2010). Role of TNF-α in wound repair in human vocal fold fibroblasts. *Laryngoscope*, 120(9), 1819–1825.

Chen, X., Xun, K., Chen, L., Wang, Y. (2009). TNF-α, a potent lipid metabolism regulator. *Cell Biochem Funct*, 27, 407–416.

Cheng, G., Zhang, X., Gao, D., Jiang, X. F., Dong, W. P. (2009). Resveratrol inhibits MMP-9 expression by up-regulating PPARα expression in an oxygen glucose deprivation-exposed neuron model. *Neurosci Lett*, 451(2), 105–108.

Chew, C. H. (2006). *Molecular regulation of PPARα gene expression by IL-6 in human hepatocarcinoma HepG2 cell line*. Ph.D. Thesis, Universiti Sains Malaysia, Malaysia.

Chew, C. H., Chew, G. S., Najimudin, N., Tengku-Muhammad, T. S. (2007). IL-6 inhibits human PPARα gene expression via CCAAT/enhancer-binding proteins in hepatocytes. *Int J Biochem Cell Biol*, 39, 1975–1986.

Chinetti, G., Fruchart, J. C., Staels, B. (2000). Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. *Inflamm Res*, 49, 497–505.

Chinetti, G., Griglio, S., Antonucci, M., Torra, I. P., Delerive, P., Majd, Z., Fruchart, J. C., Chapman, J., Najib, J., Staels, B. (1998). Activation of PPARα and γ induces apoptosis of human monocyte-derived macrophages. *J Biol Chem*, 273, 25573–25580.

Chinetti, G., Lestavel, S., Bocher, V., Remaley, A. T., Neve, B., Torra, I. P., Teissier, E., Minnich, A., Jaye, M., Duverger, N., Brewer, H. B., Fruchart, J. C., Clavey, V., Staels, B. (2001). PPARα and PPARγ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med*, 7(1), 53–58.

Coussens, L. M., Werb, Z. (2002). Inflammation and cancer. *Nature*, 420(6917), 860–867.

Csiszar, A., Smith, K., Labinskyy, N., Orosz, Z., Rivera, A., Ungvari, Z. (2006). Resveratrol attenuates TNF-α-induced activation of coronary arterial endothelial cells: role of NF-κB inhibition. *AJP-Heart*, 291(4), H1694–H1699.

Cullen, S. (2009). *Molecular regulation of inflammation by the proteasome*. Ph. D. Thesis, University of Arkansas for Medical Sciences, United States.

Cuzzocrea, S., Bruscoli, S., Mazzon, E., Crisafulli, C., Donato, V., Paola, R. D., Velardi, E., Esposito, E., Nocentini, G., Riccardi, C. (2008). PPARα contributes to the anti-inflammatory activity of glucocorticoids. *Mol Pharmacol*, 73(2), 323–337.

Cvoro, A., Tatomer, D., Tee, M. K., Zogovic, T., Harris, H. A., Leitman, D. C. (2008). Selective estrogen receptor-β agonists repress transcription of proinflammatory genes. *J Immunol*, 180(1), 630–636.

Darnay, B. G., Aggarwal, B. B. (1997). Early events in TNF signalling: a story of associations and dissociations. *J Leukoc Biol*, 61, 559–566.

Dasarathy, S. (2008). Inflammation and liver. *JPEN J Parenter Enteral Nutr*, 32(6), 660–666.

Davis, R. E., Brown, K. D., Siebenlist, U., Staudt, L. M. (2001). Constitutive NF-κB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *JEM*, 194(12), 1861–1874.

Daynes, R., Jones, D. (2002). Emerging roles of PPARs in inflammation and immunity. *Nat Rev Immunol*, 2, 748–759.

De Bosscher, K., Vanden Berghe, W., Vermeulen, L., Plaisance, S., Boone, E., Haegeman, G. (2000). Glucocorticoids repress NF-kappaB-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. *Proc Natl Acad Sci USA*, 97, 3919–3924.

De Ferrari, D., Aitken, S. (2006). Mining housekeeping genes with a Naïve Bayes classifier. *BMC Genomics*, 7, 277.

Dealtry, G. B., Naylor, M. S., Fiers, W., Balkwill, F. R. (1987). The effect of recombinant human necrosis factor on growth and macromolecular synthesis of human epithelial cells. *Exp Cell Res*, 170, 428–438.

Dejardin, E. (2006). The alternative NF-κB pathway from biochemistry to biology: pitfalls and promises for future drug development. *Biochem Pharmacol*, 72(9), 1161–1179.

Delerive, P., De Bosscher, K., Besnard, S., Vanden Berghe, W., Peters, J. M., Gonzalez, F. J., Fruchart, J. C., Tedgui, A., Haegeman, G., Staels, B. (1999a). PPARα negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-κB and AP-1. *J Biol Chem*, 274(5), 32048–32054.

Delerive, P., De Bosscher, K., Vanden Berghe, W., Fruchart, J. C., Haegeman, G., Staels, B. (2002). DNA binding-independent induction of IκBα gene transcription by PPARα. *Mol Endocrinol*, 16(5), 1029–1039.

Delerive, P., Fruchart, J. C., Staels, B., (2001). Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol*, 169, 453–459.

Delerive, P., Gervois, P., Fruchart, J. C., Staels, B. (2000). Induction of IκBα expression as a mechanism contributing to the anti-inflammatory activities of PPARα activators. *J Biol Chem*, 275(47), 36703–36707.

Delerive, P., Martin-Nizard, F., Chinetti, G., Trottein, F., Fruchart, J. C., Najib, J., Duriez, P., Staels, B. (1999b). PPAR activators inhibit thrombin- induced endothelin-1 production in human vascular endothelial cells by inhibiting the activator protein-1 signaling pathway. *Circ Res*, 85(5), 394-402.

Demarchi, F., Bertoli, C., Sandy, P., Schneider, C. (2003). Glycogen synthase kinase-3 beta regulates NF-κB1/p105 stability. *J Biol Chem*, 278(41), 39583–39590.

Demchenko, Y. N., Glebov, O. K., Zingone, A., Keats, J. J., Bergsagel, P. L., Kuehl, W. M. (2010). Classical and/or alternative NF-κB pathway activation in multiple myeloma. *Blood*, 115(17), 3541–3552.

Deng, W. G., Wu, K. K. (2003). Regulation of inducible nitric oxide synthase expression by p300 and p50 acetylation. *J Immunol*, 171, 6581–6588.

Dentin, R., Pégorier, J. P., Benhamed, F., Foufelle, F., Ferré, P., Fauveau, V., Magnuson, M. A., Girard, J., Postic, C. (2004). Hepatic glucokinase is required for the synergistic action of ChREBP and SREBP-1c on glycolytic and lipogenic gene expression. *J Biol Chem*, 279(19), 20314–20326.

Derudder, E., Dejardin, E., Pritchard, L. L., Green, D. R., Kormer, M., Baud, V. (2003). RelB/p50 dimers are differentially regulated by tumor necrosis factor-alpha and lymphotoxin-beta receptor activation: critical roles for p100. *J Biol Chem*, 278(26), 23278–23284.

Desvergne, B., Michalik, L., Wahli, W. (2004). Be fit or be sick: peroxisome proliferator-activated receptors are down the road. *Mol Endocrinol*, 18, 1321–1332.

Desvergne, B., Wahli, W. (1999). PPARs: nuclear control of metabolism. *Endocrinol Rev*, 20, 649–688.

Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J., Wahli, W. (1996). The PPARα-leukotriene B4 pathway to inflammation control. *Nature*, 384, 39–43.

Dheda, K., Huggett, J. F., Bustin, S. A., Johnson, M. A., Rook, G., Zumla, A. (2004). Validation of housekeeping genes for normalising RNA expression in Real-Time PCR. *Biotechniques*, 37, 112–119.

Dhingra, S., Sharma, A. K., Arora, R. C., Slezak, J., Singal, P. K. (2009). IL-10 attenuates TNF-α-induced NF-κB pathway activation and cardiomyocyte apoptosis. *Cardiovascular Res*, 82, 59–66.

Diep, Q. N., Touyz, R. M., Schiffrin, E. L. (2000). Docosahexaenoic acid, a PPARα ligand, induces apoptosis in vascular smooth muscle cells by stimulation of p38 mitogen-activated protein kinase. *Hypertension*, 36, 851–855.

Diradourian, C., Le May, C., Caüzac, M., Girard, J., Burnol, A. F., Pégorier, J. P. (2008). Involvement of ZIP/p62 in the regulation of PPARα transcriptional activity by p38-MAPK. *Biochim Biophys Acta*, 1781(5), 239–244.

Doi, T. S., Marino, M. W., Takahashi, T., Yoshida, T., Sakakura, T., Old, L. J., Obata, Y. (1999). Absence of TNF rescues RelA-deficient mice from embryonic lethality. *Proc Natl Acad Sci USA*, 96, 2994–2999.

Dowell, P., Ishmael, J. E., Avram, D., Peterson, V. J., Nevrivy, D. J., Leid, M. (1999). Identification of nuclear receptor corepressor as a PPARα interacting protein. *J Biol Chem*, 274, 15901–15907.

Dreyer, C., Keller, H., Mahfoudi, A., Laudet, V., Krey, G., Wahli, W. (1993). Positive regulation of the peroxisomal beta-oxidation pathway by fatty acids through activation of PPAR. *Biol Cell*. 77, 67–76.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., Wahli, W. (1992). Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. *Cell*, 68(5), 879–887.

Drosatos, K., Drosatos-Tampakaki, Z., Khan, R., Homma, S., Schulze, P. C., Zannis, V. I., Goldberg, I. J. (2011). Inhibition of c-Jun-N-terminal kinase increases cardiac PPARα expression and fatty acid oxidation and prevents lipopolysaccharide-induced heart dysfunction. *J Biol Chem*, 286(42), 36331–36339.

Ea, C. K., Baltimore, D. (2009). Regulation of NF-κB activity through lysine monomethylation of p65. *Proc Natl Acad Sci USA*, 106(45), 18972–18977.

Evans, M., Anderson, R. A., Graham, J., Ellis, G. R., Morris, K., Davies, S., Jackson, S. K., Lewis, M. J., Frenneaux, M. P., Rees, A. (2000). Ciprofibrate therapy improves endothelial function and reduces postprandial lipemia and oxidative stress in type 2 diabetes mellitus. *Circulation*, 101(15), 1773–1779.

Evans, R. M., Barish, G. D., Wang, Y. X. (2004). PPARs and the complex journey to obesity. *Nat Med*, 10, 355–361.

Faiola, B., Falls, J. G., Peterson, R. A., Bordelon, N. R., Brodie, T. A., Cummings, C. A., Romach, E. H., Miller, R. T. (2008). PPARα, more than PPARδ, mediates the hepatic and skeletal muscle alterations induced by the PPAR agonist GW0742. *Toxicol Sci*, 105(2), 384–394.

Feige, J. N., Gelman, L., Michalik, L., Desvergne, B., Wahli, W. (2006). From molecular action to physiological outputs: PPARs are nuclear receptors at the crossroads of key cellular functions. *Prog Lipid Res*, 45(2), 120–159.

Forman, B. M., Chen, J., Evans, R. M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for PPARα and δ. *Proc Natl Acad Sci USA*, 94, 4312–4317.

Francis, G. A., Fayard, E., Picard, F., Auwerx, J. (2003). Nuclear receptors and the control of metabolism. *Annu Rev Physiol*, 65, 261–311.

Frevel, M. A., Bakheet, T., Silva, A. M., Hissong, J. G., Khabar, K. S., Williams, B. R. (2003). p38 MAPK-dependent and -independent signalling of mRNA stability of AU-rich element-containing transcripts. *Mol Cell Biol*, 23(2), 425–436.

Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez De Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G., Piomelli, D. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPARα. *Nature*, 425, 90–93.

Fujita, T., Nolan, G. P, Ghosh, S., Baltimore, D. (1992). Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. *Genes Dev*, 6, 775–787.

Fujita, T., Nolan, G. P., Liou, H. C., Scott, M. L., Baltimore, D. (1993). The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-κB p50 homodimers. *Genes Dev*, 1354–1363.

Gearing, K. L., Gottlicher, M., Teboul, M., Widmark, E., Gustafsson, J. A. (1993). Interaction of the peroxisome-proliferator-activated receptor and retinoid X receptor. *Proc Natl Acad Sci USA*, 90(4), 1440–1444.

Geisler, F., Algül, H., Paxian, S., Schmid, R. M. (2007). Genetic inactivation of RelA/p65 sensitizes adult mouse hepatocytes to TNF-induced apoptosis *in vivo* and *in vitro*. *Gastroenterology*, 132(7), 2489–2503.

Gelman, L., Michalik, L., Desvergne, B., Wahli, W. (2005). Kinase signaling cascades that modulate PPARs. *Curr Opin Cell Biol*, 17(2), 216–222.

Geng, H., Wittwer, T., Dittrich-Breiholz, O., Kracht, M., Schmitz, M. L. (2009). Phosphorylation of NF-κB p65 at Ser468 controls its COMMD1-dependent ubiquitination and target gene-specific proteasomal elimination. *EMBO Rep*, 10(4), 381–386.

Geng, Y. J, Wu, Q., Muszynski, M., Hansson, G. K., Libby, P. (1996). Apoptosis of vascular smooth muscle cells induced by *in vivo* stimulation with IFN-γ, TNF-α, IL-1β. *Arterioscler Thromb Vasc Biol*, 16, 19–27.

Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., Collins, T. (1997). CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc Natl Acad Sci USA*, 94, 2927–2932.

Gervois, P., Kleemann, R., Pilon, A., Percevault, F., Koenig, W., Staels, B., Kooistra, T. (2004). Global suppression of IL-6-induced acute phase response gene expression after chronic in vivo treatment with the PPARα activator fenofibrate. *J Biol Chem*, 279, 16154–16160.

Gervois, P., Vu-Dac, N., Kleemann, R., Kockx, M., Dubois, G., Laine, B., Kosykh, V., Fruchart, J. C., Kooistra, T., Staels, B. (2001). Negative regulation of human fibrinogen gene expression by PPARα agonists via inhibition of CCAAT box/enhancer-binding protein β. *J Biol Chem*, 276, 33471–33477.

Ghosh, C. C., Ramaswami, S., Juvekar, A., Vu, H. Y., Galdieri, L., Davidson, D., Vancurova, I. (2010). Gene-specific repression of proinflammatory cytokines in stimulated human macrophages by nuclear IκBα. *J Immunol*, 185, 3685–3693.

Gilmore, T. D. (2006). Introduction to NF-κB: players, pathways, perspectives. *Oncogene*, 25, 6680–6684.

Glare, E. M., Divjak, M., Bailey, M. J., Walters, E. H. (2002). Beta-actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax*, 57(9), 765–770.

Glass, C. K., Ogawa, S. (2006). Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat Rev Immunol*, 6, 44–55.

Glass, C. K., Rosenfeld, M. G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev*, 14, 121–141.

Glosli, H., Gudbrandsen, Q. A., Mullen, A. J., Halvorsen, B., Røst, T. H., Wergedahi, H., Prydz, H., Aukrust, P., Berge, R. K. (2005). Down-regulated expression of PPARα target genes, reduced fatty acid oxidation and altered fatty acid composition in the liver of mice transgenic for hTNFα. *Biochim Biophys Acta*, 1734(3), 235–246.

Goetze, S., Eilers, F., Bungenstock, A., Kintscher, U., Stawowy, P., Blaschke, F., Graf, K., Law, R. E., Fleck, E., Grafe, M. (2002). PPAR activators inhibit endothelial cell migration by targeting Akt. *Biochem Biophys Res Commun*, 293, 1431–1437.

Gonzalez, F. J. (1997). The role of peroxisome proliferator activated receptor alpha in peroxisome proliferation, physiological homeostasis, and chemical carcinogenesis. *Adv Exp Med Biol*, 422, 109–125.

Goto, M., Yoshioka, T., Young, R. I., Battelino, T., Anderson, C. L., Zeller, W. P. (1997). A sublethal dose of LPS to pregnant rats induces TNF-α tolerance in their 0-day-old offspring. *Am J Physiol*, 273, R1158–R1162.

Göttlicher, M., Widmark, E., Li, Q., Gustafsson, J. A. (1992). Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc Natl Acad Sci USA*, 89(10), 4653–4657.

Grilli, M., Chiu, J. J., Lenardo, M. J. (1993) NF-*κ*B and Rel: participants in a multiform transcriptional regulatory system. *Int Rev Cytol*, 143, 1–62.

Grunfeld, C., Zhao, C., Fuller, J., Pollack, A., Moser, A., Friedman, J., Feingold, K. R. (1996). Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters. *J Clin Invest*, 97, 2152–2157.

Gu, P., Yang, J., Wang, J., Young, M. J., Klassen, H. (2009). Sequential changes in the gene expression profile of murine retinal progenitor cells during the induction of differentiation. *Mol Vis*, 15, 2111–2122.

Guan, Y., Zhang, Y., Davis, L., Breyer, M. D. (1997). Expression of PPARs in urinary tract of rabbits and humans. *Am J Physiol*, 273, 1013–1022.

Haas, M. J., Horani, M., Mreyoud, A., Plummer, B., Wong, N. C., Mooradian, A. D. (2003). Suppression of apolipoprotein AI gene expression in HepG2 cells by TNF-α and IL-1β. *Biochim Biophys Acta*, 1623(2–3), 120–128.

Han, S. S., Yun, H., Son, D. J., Tompkins, V. S., Peng, L., Chung, S. T., Kim, J. S., Park, E. S., Janz, S. (2010). NF-*κ*B/STAT3/PI3K signalling crosstalk in iMyc[Eμ] B lymphoma. *Mol Cancer*, 9, 97.

Hanley, K., Jiang, Y., He, S. S., Friedman, M., Elias, P. M., Bikle, D. D., Williams, M. L., Feingold, K. R. (1998). Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPARα. *J Invest Dermatol*, 110, 368–375.

Hanley, K., Komuves, L. G., Ng, D. C., Schoonjans, K., He, S. S., Lau, P., Bikle, D. D. Williams, M. L., Elias, P. M., Auwerx, J., Feingold, K. R. (2000). Farnesol stimulates differentiation in epidermal keratinocytes via PPARα. *J Biol Chem*, 275, 11484–11491.

Hansmannel, F., Clemencet, M. C., Le Jossic-Corcos, C., Osumi, T., Latruffe, N., Nicolas-Frances, V. (2003). Functional characterization of a peroxisome proliferator response-element located in the intron 3 of rat peroxisomal thiolase B gene. *Biochem Biophys Res Commun*, 311, 149–155.

Harano, Y., Yasui, K., Toyama, T., Nakajima, T., Mitsuyoshi, H., Mimani, M., Hirasawa, T., Itoh, Y., Okanoue, T. (2006). Fenofibrate, a PPARα agonist, reduces hepatic steatosis and lipid peroxidation in fatty liver Shionogi mice with hereditary fatty liver. *Liver Int*, 26(5), 613–620.

Haridas, V., Darnay, B. G., Natarajan, K., Heller, R., Aggarwal, B. B. (1998). Overexpression of the p80 TNF receptor leads to TNF-dependent apoptosis, NF-κB activation and c-Jun kinase activation. *J Immunol*, 160, 3152–3162.

Hashimoto, T., Cook, W. S., Qi, C., Yeldandi, A. V., Reddy, J. K., Rao, M. S. (2000). Defect in PPARα inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *J Biol Chem*, 275, 28918–28928.

Hay, R. T., Vuillard, L., Desterro, J. M. P., Rodriguez, M. S. (1999). Control of NF-κB transcriptional activation by signal induced proteolysis of IκBα. *Phil Trans R Soc Lond B*, 354, 1601–1609.

Hayden, M. S., Ghosh, S. (2004). Signalling to NF-κB. *Genes Dev*, 18, 2195–2224.

Hayden, M. S., Ghosh, S. (2008). Shared principles in NF-κB Signalling. *Cell*, 132, 344–362.

Hayden, M. S., Ghosh, S. (2011). NF-κB in immunobiology. *Cell Res*, 21, 223–244.

He, G., Karin, M. (2011). NF-κB and STAT3 – key players in liver inflammation and cancer. *Cell Res*, 21(1), 159–168.

Hi, R., Osada, S., Yumoto, N., Osumi, T. (1999). Characterisation of amino-terminal activation domain of PPARα. Importance of α-helical structure in the transactivation function. *J Biol Chem*, 274, 35152–35158.

Hihi, A. K., Michalik, L., Wahli, W. (2002). PPARs: Transcriptional effectors of fatty acids and their derivates. *Cell Mol Life Sci*, 59, 790–798.

Hill, D. B., Schmidt, J., Shedlofsky, S. I., Cohen, D. A., McClain, C. J. (1995). *In vitro* TNF cytotoxicity in HepG2 liver cells. *Hepatology*, 21(4), 1114–1119.

Hirayama, M., Kato, J., Kohgo, Y., Kondo, H., Niitsu, Y., Sasaki, K., Shintani, N. (1993). Regulation of iron metabolism in HepG2 cells: a possible role for cytokines in the hepatic deposition of iron. *Hepatology*, 18, 874–880.

Hirota, A., Kawachi, Y., Itoh, K., Nakamura, Y., Xu, X. Z., Banno, T., Takahashi, T., Yamamoto, M., Otsuka, F. (2005). Ultraviolet A irradiation induces NF-E2-related factor 2 activation in dermal fibroblasts: Protective role in UVA-induced apoptosis. *J Invest Dermatol*, 124, 825–832.

Hoberg, J. E., Yeung, F., Mayo, M. W. (2004). SMRT derepression by the IκB kinase α: A prerequisite to NF-κB transcription and survival. *Mol Cell*, 16, 245–255.

Hoffmann, A., Leung, T. H., Baltimore, D. (2003). Genetic analysis of NF-κB/Rel transcription factors defines functional specificities. *EMBO J*, 22, 5530–5539.

Hoffmann, A., Natoli, G., Ghosh, G. (2006). Transcriptional regulation via the NF-κB signalling module. *Oncogene*, 25, 6706–6716.

Horwitz, B. H., Scott, M. L., Cherry, S. R., Bronson, R. T., Baltimore, D. (1997). Failure of lymphopoiesis after adoptive transfer of NF-κB-deficient fetal liver cells. *Immunity*, 6, 765–772.

Hsu, H., Xiong, J., Goeddel, D. V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappaB activation. *Cell*, 81, 495–504.

Hu, J., Nakano, H., Sakurai, H., Colburn, N. H. (2004). Insufficient p65 phosphorylation at S536 specifically contributes to the lack of NF-κB activation and transformation in resistant JB6 cells. *Carcinogenesis*, 25(10), 1991–2003.

Huang, B., Yang, X. D., Lamb, A., Chen, L. F. (2010). Posttranslational modifications of NF-κB: Another layer of regulation for NF-κB signalling pathway. *Cell Signal*, 22(9), 1282–1290.

Huang, Q., Yang, J., Lin, Y., Walker, C., Cheng, J., Liu, Z. G., Su, B. (2004). Differential regulation of IL-1 receptor and Toll-like receptor signalling by MEKK3. *Nat Immunol*, 5, 98–103.

Huang, W. C., Chen, C. C. (2005). Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. *Mol Cellular Biol*, 25(15), 6592–6602.

Hunter, T., Garrels, J. I. (1977). Characterisation of the mRNAs for alpha-, beta- and gamma-actin. *Cell*, 12, 767–781.

Hwang, M. K., Song, N. R., Kang, N. J., Lee, K. W., Lee, H. J. (2009). Activation of phosphatidylinositol 3-kinase is required for TNF-α-induced upregulation of matrix metalloproteinase-9: Its direct inhibition by quercetin. *Int J of Biochem Cell Biol*, 41(7), 1592–1600.

Inoue, H., Jiang, X. F., Katayama, T., Osada, S., Umesono, K., Namura, S. (2003). Brain protection by resveratrol and fenofibrate against stroke requires PPARα in mice. *Neurosci Lett*, 352, 203–206.

Inoue, I., Shino, K., Noji, S., Awata, T., Katayama, S. (1998). Expression of PPARα in primary cultures of human vascular endothelial cells. *Biochem Biophys Res Commun*, 246, 370–374.

Ip, E., Farrell, G., Hall, P., Robertson, G., Leclercq, I. (2004). Administration of the potent PPARα agonist, Wy-14,643, reverses nutritional fibrosis and steatohepatitis in mice. *Hepatology*, 39, 1286–1296.

Irukayama-Tomobe, Y., Miyauchi, T., Sakai, S., Kasuya, Y., Ogata, T., Takanash, M., Iemitsu, M., Sudo, T. Goto, K., Yamaguchi, I. (2004). Endothelin-1-induced cardiac hypertrophy is inhibited by activation of PPARα partly via blockade of JNK pathway. *Circulation*, 109(7), 904–910.

Israël, A. (2010). The IKK complex, a central regulator of NF-κB activation. *Cold Spring Harb Perspect Biol*, 2(3), a000158.

Issemann, I., Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, 347(6294), 645–650.

Jacque, E., Tchenio, T., Piton, G., Romeo, P. H., Baud, V. (2005). RelA repression of RelB activity induces selective gene activation downstream of TNF receptors. *Proc Natl Acad Sci USA*, 102, 14635–14640.

Jamaluddin, M., Wang, S., Boldogh, I., Tian, B., Brasier, A. R. (2007). TNF-α-induced NF-κB/Rel A Ser276 phosphorylation and enhanceosome formation on the IL-8 promoter is mediated by a reactive oxygen species (ROS)-dependent pathway. *Cell Signal*, 9, 1419–1433.

Javitt, N. B. (1990). HepG2 cells as a resource for metabolic studies: lipoprotein, cholesterol, and bile acids. *FASEB J*, 4, 161–168.

Jee, H. L., Eun-hye, J., Ilo, J. (2005). PPARα activators suppress STAT1 inflammatory signalling in lipopolysaccharide-activated rat glia. *Neuroreport*, 16(8), 829–833.

Jeong, S. J., Pise-Masison, C. A., Radonovich, M. F., Park, H. U., Brady, J. N. (2005). A novel NF-κB pathway involving IKKβ and p65/RelA Ser-536 phosphorylation results in p53 inhibition in the absence of NF-κB transcriptional activity. *J Biol Chem*, 280(11), 10326–10332.

Jepsen, K., Hermannson, O., Thandi, O., Gleiberman, A., Lunyak, V., Kurokawa, R., Kumar, V., Liu, F., Seto, E., Hedrick, S., Mandel, G., Glass, C., Rose, D., Rosenfeld, M. (2000). Combinatorial roles of the nuclear receptor corepressor in transcription and development. *Cell*, 102, 753–763.

Jochems, C. E. A., van der Valk, J. B. F., Stafleu, F. R., Baumans, V. (2002). The use of fetal bovine serum: ethical or scientific problem? *Altern Lab Anim*, 30(2), 219–227.

Jones, D. C., Ding, X., Zhang, T. Y., Daynes, R. A. (2003). Peroxisome proliferator-activated receptor alpha negatively regulates T-bet transcription through suppression of p38 mitogen-activated protein kinase activation. *J Immunol*, 171, 196–203.

Jones, D. T., Harris, A. L. (2006). Identification of novel small-molecule inhibitors of hypoxia-inducible factor-1 transactivation and DNA binding. *Mol Cancer Ther*, 5, 2193.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S. C., Heyman, R., Rose, D., Glass, C., Rosenfeld, M. (1996). A CBP integratoa complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*, 85, 403–414.

Kane, C. D., Stevens, K. A., Fischer, J. E., Haghpassand, M., Royer, L. J., Aldinger, C., Landschulzl, K. T., Zagouras, P., Bagley, S. W., Hada, W., Dullea, R., Hayward, C. M., Francone, O. L. (2009). Molecular characterization of novel and selective PPARα agonists with robust hypolipidemic activity *in vivo*. *Mol Pharmacol*, 75(2), 296–306.

Karin, M. (1999). How NF-κB is activated: the role of the IκB kinase (IKK) complex. *Oncogene*, 18, 6867–6874.

Karin, M., Lin, A. (2002). NF-κB at the crossroads of life and death. *Nat Immunol*, 3, 221–227.

Keller, H., Devchand, P. R., Perroud, M., Wahli, W. (1997). PPARα structure-function relationships derived from species-specific differences in responsiveness to hypolipidemic agents. *Biol Chem*, 378(7), 651–655.

Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., Wahli, W. (1999). PPARα mediates the adaptive response to fasting. *J Clin Invest*, 103(11), 1489–1498.

Khammanit, R., Chantakru, S., Kitiyanant, Y., Saikhun, J. (2008). Effect of serum starvation and chemical inhibitors on cell cycle synchronisation of canine dermal fibroblasts. *Theriogenology*, 70(1), 27–34.

Kim, M. S., Sweeney, T. R., Shigenaga, J. K., Chui, L. G., Moser, A., Grunfeld, C., Feingold, K. R. (2007). TNF and IL-1 decrease RXRα, PPARα, PPARγ, LXRα, and the coactivators SRC-1, PGC-1α, and PGC-1β in liver cells. *Metabolism*, 56(2), 267–279.

Kishore, N., Sommers, C., Mathialagan, S., Guzova, J., Yao, M., Hauser, S., Huynh, K., Bonar, S., Mielke, C., Albee, L., Weier, R., Graneto, M., Hanau, C., Perry, T., Tripp, C. S. (2003). A selective IKK-2 inhibitor blocks NF-κB-dependent gene expression in IL-1β-stimulated synovial fibroblasts. *J Biol Chem*, 278, 32861–32871.

Kleemann, R., Gervois, P., Verschuren, L., Staels, B., Princen, H. M., Kooistra, T. (2003). Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing p50-NF-κB-C/EBPβ complex formation. *Blood*, 101, 545–551.

Kleemann, R., Verschuren, L., De Rooij, B. J., Lindeman, J., De Maat, M. M., Szalai, A. J., Princen, H. M., Kooistra, T. (2004). Evidence for anti-inflammatory activity of statins and PPARα activators in human C-reactive protein transgenic mice *in vivo* and in cultured human hepatocytes *in vitro*. *Blood*, 103, 4188–4194.

Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K., Evans, R. M. (1994). Differential expression and activation of a family of murine PPARs. *Proc Natl Acad Sci USA*, 91(15), 7355–7359.

Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., Lehmann, J. M. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with PPARα and γ. *Proc Natl Acad Sci USA*, 94(9), 4318–4323.

Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., Evans, R. M., (1992). Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature*, 358, 771–774.

Knight, B. L., Hebbach, A., Hauton, D., Brown, A. M., Wiggins, D., Patel, D. D. (2005). A role of PPARα in the control of SREBP activity and lipid synthesis in the liver. *Biochem J*, 389, 413–421.

Kobori, M., Yang, Z., Gong, D., Heissmeyer, V., Zhu, H., Jung, K., Gakidis, M. A., Rao, A., Sekine, T., Ikegami, F., Yuan, C., Yuan, J. (2004). Wedelolactone suppresses LPS-induced caspase-11 expression by directly inhibiting the IKK complex. *Cell Death Differ*, 11, 123–130.

König, B., Koch, A., Spielmann, J., Hilgenfeld, C., Stangl, G. I., Eder, K. (2007). Activation of PPARα lowers synthesis and concentration of cholesterol by reduction of nuclear SREBP-2. *Biochem Pharmacol*, 73, 574–585.

Kunsch, C., Rosen, C. A. (1993). NF-κB subunit-specific regulation of the IL-8 promoter. *Mol Cell Biol*, 13, 6137–6146.

Ladias, J. A., Hadzopoulou-Cladaras, M., Kardassis, D., Cardot, P., Cheng, J., Zannis, V., Cladaras, C. (1992). Transcriptional regulation of human apolipoprotein genes ApoB, ApoCIII, and ApoAII by members of the steroid hormone receptor superfamily HNF4, ARP-1, EAR-2, and EAR-3. *J Biol Chem*, 267(22), 15849–15860.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685.

Lang, V., Janzen, J., Fischer, G. Z., Soneji, Y., Beinke, S., Salmeron, A., Allen, H., Hay, R. T., Ben-Neriah, Y., Ley, S. C. (2003). βTrCP-mediated proteolysis of NF-κB1 p105 requires phosphorylation of p105 serines 927 and 932. *Mol Cell Biol*, 23(1), 402–413.

Lazennec, G., Canaple, L., Saugy, D., Wahli, W. (2000). Activation of PPARs by their ligands and protein kinase A activators. *Mol Endocrinol*, 14, 1962–1975.

Lee, S. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kroetz, D. L., Fernandez-Salguero, P. M., Westphal, H., Gonzalez, F. J. (1995). Targeted disruption of the alpha isoform of the PPAR gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol*, 15, 3012–3022.

Lefebvre, P., Chinetti, G., Fruchart, J. C., Staels, B. (2006). Sorting out the roles of PPARα in energy metabolism and vascular homeostasis. *J Clin Invest*, 116(3), 571–580.

Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M., Kliewer, S. A. (1997). PPARα and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem*, 272, 3406–3410.

Leinonen, E., Hurt-Camejo, E., Wiklund, O., Hultén, L. M., Hiukka, A., Taskinen, M. R. (2003). Insulin resistance and adiposity correlate with acute-phase reaction and soluble cell adhesion molecules in type 2 diabetes. *Atherosclerosis*, 166(2), 387–394.

Leist, M., Gantner, F., Bohlinger, I., Germann, P. G., Tiegs, G., Wendel, A. (1994). Murine hepatocyte apoptosis induced *in vitro* and *in vivo* by TNF-α requires transcriptional arrest. *J Immunol*, 153, 1778–1788.

Leist, M., Gantner, F., Kunstle, G., Wendel, A. (1998). Cytokine-mediated hepatic apoptosis. *Res Physiol Biochem Pharmacol*, 133, 109–155.

Lemberger, T., Desvergne, B., Wahli, W. (1996). Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. *Annu Rev Cell Dev Biol*, 12, 335–363.

Lenardo, M. J., Baltimore, D. (1989). NF-κB: A pleiotropic mediator of inducible and tissue-specific gene control. *Cell*, 58(2), 227–229.

Leuenberger, N., Pradervand, S., Wahli, W. (2009). Sumoylated PPARα mediates sex-specific gene repression and protects the liver from estrogen-induced toxicity in mice. *J Clin Invest*, 119(10), 3138–3148.

Li, Q., Verma, I. M. (2002). NF-κB regulation in the immune system. *Nat Rev Immunol*, 2, 725–734.

Li, Y. D., Ye, B. Q., Zheng, S. X., Wang, J. T., Wang, J. G., Chen, M., Liu, J. G., Pei, X. H., Wang, L. J., Lin, Z. X., Gupta, K., Mackman, N., Slungaard, A., Key, N. S., Geng, J. G. (2009). NF-κB transcription factor p50 critically regulates tissue factor in deep vein thrombosis. *J Biol Chem*, 284, 4473–4483.

Li, Z., Yang, L., Wang, J., Shi, W., Pawar, R. A., Liu, Y., Xu, C., Cong, W., Hu, Q., Lu, T., Xia, F., Guo, W., Zhao, M., Zhang, Y. (2010). Beta-Actin is a useful internal control for tissue-specific gene expression studies using quantitative real-time PCR in the half-smooth tongue sole *Cynoglossus semilaevis* challenged with LPS or *Vibrio anguillarum*. *Fish Shellfish Immunol*, 29(1), 83–89.

Liang, C., Zhang, M., Sun, S. C. (2006). β-TrCP binding and processing of NF-κB2/p100 involve its phosphorylation at serines 866 and 870. *Cell Signal*, 18, 1309–1317.

Lin, A. (2003). Activation of the JNK signalling pathway: breaking the brake on apoptosis. *Bioessays*, 25, 1–8.

Liu, H., Sidiropoulos, P., Song, G., Pagliari, L. J., Birrer, M. J., Stein, B., Anrather, J., Pope, R. M. (2000). TNF-α gene expression in macrophages: regulation by NF-κB is independent of c-Jun or C/EBPβ. *J Immunol*, 164, 4277–4285.

Liu, S. F., Malik, A. B. (2006). NF-κB activation as a pathological mechanism of septic shock and inflammation. *AJP-Lung Physiol*, 290(4), L622–L645.

Locksley, R. M., Killeen, N., Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*, 104, 487–501.

Maalouf, S. W., Talhouk, R. S., Schanbacher, F. L. (2010). Inflammatory responses in epithelia: endotoxin-induced IL-6 secretion and iNOS/NO production are differentially regulated in mouse mammary epithelial cells. *J Inflamm (Lond)*, 7, 58.

MacEwan, D. J. (2002). TNF receptor subtype signalling: differences and cellular consequences. *Cell Signal*, 14, 477–492.

Madrid, L. V., Mayo, M. W., Reuther, J. Y., Baldwin A. S. Jr. (2001). Akt stimulates the transactivation potential of the RelA/p65 subunit of NF-κB through utilization of the IκB kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem*, 276, 18934–18940.

Mandard, S., Muller, M., Kersten, S. (2004). Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci*, 61, 393–416.

Mandrekar, P., Szabo, G. (2011). Molecular pathology of liver diseases. *Inflammation and liver injury*. 5(4), 411–425.

Marui, N., Medford, R. M., Ahmad, M. (2005). Activation of RelA homodimers by TNF- α: a possible transcriptional activator in human vascular endothelial cells. *Biochem J*, 390, 317–324.

Marx, N., Sukhova, G. K., Collins, T., Libby, P., Plutzky, J. (1999). PPARα activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells. *Circulation*, 99, 3125–3131.

Matsuura, F., Oku, H., Koseki, M., Sandoval, J. C., Yuasa-Kawase, M., Tsubakio-Yamamoto, K., Masuda, D., Maeda, N., Tsujii, K., Ishigami, M., Nishida, M., Hirano, K., Kihara, S., Hori, M., Shimomura, I., Yamashita, S. (2007). Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver. *Biochem Biophys Res Commun*, 358(4), 1091–1095.

Mattioli, I., Geng, H., Sebald, A., Hodel, M., Bucher, C., Kracht, M., Schmitz, M. L. (2006). Inducible phosphorylation of NF-κB p65 at serine 468 by T cell constimulation is mediated by IKKε. *J Biol Chem*, 281, 6175–6183.

McKenna, N. J., O'Malley, B. W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell*, 108, 465–474.

Mersch-Sundermann, V., Knasmüller, S., Wu, X. J., Darroudi, F., Kassie, F. (2004). Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology*, 198(1–3), 329–340.

Michalik, L., Desvergne, B., Tan, N. S., Basu-Modak, S., Escher, P., Rieusset, J., Peters, J. M., Kaya, G., Gonzalez, F. J., Zakany, J., Metzger, D., Chambon, P., Duboule, D., Wahli, W. (2001). Impaired skin wound healing in PPARα and PPARβ mutant mice. *J Cell Biol*, 154(4), 799–814.

Michaud, S. E., Renier, G. (2001). Direct regulatory effect of fatty acids on macrophage lipoprotein lipase: Potential role of PPARs. *Diabetes*, 50, 660–666.

Milosavljevic, D., Griglio, S., Le Naour, G., Chapman, M. J. (2001). Preferential reduction of very low density lipoprotein-1 particle number by fenofibrate in type IIB hyperlipidemia: consequences for lipid accumulation in human monocyte-derived macrophages. *Atherosclerosis*, 155, 251–260.

Mittra, S., Sangle, G., Tandon, R., Sharma, S., Roy, S., Khanna, V., Gupta, A., Sattigeri, J., Sharma, L., Priyadarsiny, P., Khattar, S. K., Bora, R. S., Saini, K. S., Bansal, V. S. (2007). Increase in weight induced by muraglitazar, a dual PPARα/γ agonist, in db/db mice: adipogenesis/or oedema? *Br J Pharmacol*, 150(4), 480–487.

Mogilenko, D. A., Dizhe, E. B., Shavva, V. S., Lapikov, I. A., Orlov, S. V., Perevozchikov, A. P. (2009). Role of the nuclear receptors HNF4α, PPARα, and LXRs in the TNFα-mediated inhibition of human apolipoprotein A-I gene expression in HepG2 cells. *Biochemistry*, 48(50), 11950–11960.

Mohamed, M. R., Rahman, M. M., Rice, A., Moyer, R. W., Werden, S. J., McFadden, G. (2009). Cowpox virus expresses a novel ankyrin repeat NF-κB inhibitor that controls inflammatory cell influx into virus-infected tissues and is critical for virus pathogenesis. *J Virol*, 83(18), 9223–9236.

Molin, C., Jauhiainen, A., Warringer, J., Nerman, O., Sunnerhagen, P. (2009). mRNA stability changes precede changes in steady-state mRNA amounts during hyperosmotic stress. *RNA*, 15(4), 600–614.

Moras, D., Gronemeyer, H. (1998). The nuclear receptor ligand-binding domain: Structure and function. *Curr Opin Cell Biol*, 10, 384–391.

Moreno, R., Sobotzik, J. M., Schultz, C., Schmitz, M. L. (2010). Specification of the NF-κB transcriptional response by p65 phosphorylation and TNF-induced nuclear translocation of IKKε. *Nucl Acids Res*, 38(18), 6029–6044.

Morishima, A., Ohkubo, N., Maeda, N., Miki, T., Mitsuda, N. (2003). NF-κB regulates plasma apolipoprotein A-I and high density lipoprotein cholesterol through inhibition of PPARα. *J Biol Chem*, 278(40), 38188–38193.

Mukhopadhyay, A., Suttles, J., Stout, R. D, Aggarwal, B. B. (2001). Genetic deletion of the TNFR p60 or p80 abrogates ligand-mediated activation of NF-κB and of MAPKs in macrophages. *J Biol Chem*, 276(34), 31906–31912.

Nagata, S., Golstein, P. (1995). The Fas death factor. *Science*, 267, 1449–1456.

Naumann, M., Scheidereit, C. (1994). Activation of NF-κB *in vivo* is regulated by multiple phosphorylations. *EMBO J*, 13, 4597–4607.

Neumann, M., Grieshammer, T., Chuvpilo, S., Kneitz, B., Lohoff, M., Schimpl, A., Franza, B. R. Jr., Serfling, E. (1995). RelA/p65 is a molecular target for the immunosuppressive action of protein kinase A. *EMBO J*, 14, 1991–2004.

Neumann, M., Naumann, M. (2007). Beyond IκBs: Alternative regulation of NF-κB activity. *FASEB J*, 21, 2642–2654.

Ng, D. L., Tie, S. W., Ong, P. C., Lim, W. S., Tengku-Muhammad, T. S., Choo, Q. C., Chew, C. H. (2011). Rapamycin pre-treatment abrogates TNF-α down-regulatory effects on LXR-α and PXR mRNA expression via inhibition of c-Jun N-terminal kinase 1 (JNK1) activation in HepG2 cells. *J Biotech*, 14(3).

Nishikori, M. (2005). Classical and alternative NF-κB activation pathways and their roles in lymphoid malignancies. *J Clin Exp Hematopathol*, 45(1), 15–24.

Nowak, D. E., Tian, B., Jamaluddin, M., Boldogh, I., Vergara, L. A., Choudhary, S., Brasier, A. R. (2008). RelA Ser276 phosphorylation is required for activation of a subset of NF-κB-dependent genes by recruiting cyclin-dependent kinase 9/cyclin T1 complexes. *Mol Cell Biol*, 28(11), 3623–3638.

O'Dea, E., Hoffmann, A. (2009). The regulatory logic of the NF-κB signaling system. *Cold Spring Harb Perspect Biol*, 2(1), a000216.

O'Hara, A., Lim, F. L., Mazzatti, D. J., Trayhum, P. (2012). Stimulation of inflammatory gene expression in human preadipocytes by macrophage-conditioned medium: Upregulation of IL-6 production by macrophage-derived IL-1β. *Mol Cell Endocrinol*, 349(2), 239–247.

O'Mahony, Montano, A. M., Van Beneden, K., Chen, L. F., Greene, W. C. (2004). Human T-cell lymphotropic virus type 1 tax induction of biologically active NF-κB requires IκB kinase-1-mediated phosphorylation of RelA/p65. *J Biol Chem*, 279, 18137–18145.

Oeckinghaus, A., Ghosh, S. (2009). The NF-κB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol*, 1(4), a000034.

Ogawa, S., Lozach, J., Jepsen, K., Sawka-Verhelle, D., Perissi, V., Sasik, R., Rose, D. W., Johnson, R. S., Rosenfeld, M. G., Glass, C. K. (2004). A nuclear receptor corepressor transcriptional checkpoint controlling activator protein 1-dependent gene networks required for macrophage activation. *Proc Natl Acad Sci USA*, 101, 14461–14466.

Okayasu, T., Tomizawa, A., Suzuki, K., Manaka, K., Hattori, Y. (2008). PPARα activators upregulate eNOS activity and inhibit cytokine-induced NF-κB activation through AMP-activated protein kinase activation. *Life Sci*, 82(15–16), 884–891.

Okazaki, T., Sakon, S., Sasazuki, T., Sakurai, H., Doi, H., Yagita, H., Okumura, K., Nakano, H. (2003). Phosphorylation of serine-276 is essential for p65 NF-κB subunit-dependent cellular responses. *Biochem Biophys Res Commun*, 300, 807–812.

Orian, A., Gonen, H., Bercovich, B., Faierman, I., Evtan, E., Israël, A., Mercurio, F., Iwai, K., Schwartz, A. L., Ciechanover, A. (2000). SCF(beta)(-TrCP) ubiquitin ligase-mediated processing of NF-κB p105 requires phosphorylation of its C-terminus by IκB kinase. *EMBO J*, 19(11), 2580–2591.

Orlov, S. V., Mogilenko, D. A., Shavva, V. S., Dizhe, E. B., Ignatovich, I. A., Perevozchikov, A. P. (2010). Effect of TNF-α on activities of different promoters of human apolipoprotein A-I gene. *Biochem Biophys Res Commun*, 398(2), 224–230.

Ostlund, C., Worman, H. J. (2003). Nuclear envelope proteins and neuromuscular diseases. *Muscle Nerve*, 27, 393–406.

Pahl, H. L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene*, 18, 6853–6866.

Palomer, X., Álvarez-Guardia, D., Rodríguez-Calvol, R., Coll, T., Laguna, J. C., Davidson, M. M., Chan, T. O., Feldman, A. M., Vázquez-Carreral, M. (2009). TNF-α reduces PGC-1α expression through NF-κB and p38 MAPK leading to increased glucose oxidation in a human cardiac cell model. *Cardiovasc Res*, 81, 703–712.

Pandey, A. K., Munjal, N., Datta, M. (2010). Gene expression profiling and network analysis reveals lipid and steroid metabolism to be the most favoured by TNFα in HepG2 cells. *PLoS One*, 5(2), e9063.

Pang, L., Nie, M., Corbett, L., Knox, A. (2003). Cyclooxygenase-2 expression by nonsteroidal anti-inflammatory drugs in human airway smooth muscle cells: Role of peroxisome proliferatoractivated receptors. *J Immunol*, 170, 1043–1051.

Papa, S., Bubici, C., Zazzeroni, F., Franzoso, G. (2009). Mechanisms of liver disease: The crosstalk between the NF-κB and JNK pathways. *Biol Chem*, 390(10), 965–976.

Parent, R., Kolippakkam, D., Booth, G., Beretta, L. (2007). Mammalian target of rapamycin activation impairs hepatocytic differentiation and targets genes moderating lipid homeostasis and hepatocellular growth. *Cancer Res*, 67, 4337.

Park, C. W., Zhang, Y., Zhang, X., Wu, J., Chen, J., Cha, D. R., Su, D., Hwang, M. –T., Fan, X., Davis, L., Striker, G., Zheng, F., Breyer, M., Guan, Y. (2006). PPARα agonist fenofibrate improves diabetic nephropathy in db/db mice. *Kidney Int*, 69, 1511–1517.

Pascual, G., Glass, C. K. (2006). Nuclear receptors versus inflammation: mechanisms of transrepression. *Trends Endocrinol Metab*, 17, 321–327.

Perkins, N. D. (2006). Post-translational modifications regulating the activity and function of the NF-κB pathway. *Oncogene*, 25(51), 6717–6730.

Peters, J. M., Hennuyer, N., Staels, B., Fruchart, J. C., Fievet, C., Gonzalez, F. J., Auwerx, J. (1997). Alterations in lipoprotein metabolism in PPARα-deficient mice. *J Biol Chem*, 272, 27307–27312.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in Real-Time RT-PCR. *Nucl Acids Res*, 29, 2002–2007.

Phulwani, N. K., Essen, N., Syed, M. M., Kielian, T. (2008). TLR2 expression in astrocytes is induced by TNF-α- and NF-κB-dependent pathways. *J Immunol*, 181(6), 3841–3849.

Pierce, R. H., Campbell, J. S., Stepenson, A. B., Franklin, C. C., Chaisson, M., Poot, M., Kavanagh, T. J., Rabinovitch, P. S., Fausto, N. (2000). Disruption of redox homeostasis in TNF-induced apoptosis in a murine hepatocyte cell line. *Am J Pathol*, 157(1), 221–236.

Planavila, A., Laguna, J. C., Vazquez-Carrera, M. (2005a). NF-kappaB activation leads to down-regulation of fatty acid oxidation during cardiac hypertrophy. *J Biol Chem*, 280, 17464–17471.

Planavila, A., Rodriguez-Calvo, R., Jove, M., Michalik, L., Wahli, W., Laguna, J. C., Vazquez-Carrera, M. (2005b). PPARβ/δ activation inhibits hypertrophy in neonatal rat cardiomyocytes. *Cardiovasc Res*, 65, 832–841.

Pourcet, B., Pineda-Torra, I., Derudas, B., Staels, B., Glineur, C. (2010). SUMOylation of human PPARα inhibits its trans-activity through the recruitment of the nuclear corepressor NCoR. *J Biol Chem*, 285, 5983–5992.

Poynter, M. E. and Daynes, R. A. (1998). PPARα activation modulates cellular redox status, represses NF-κB signalling, and reduces inflammatory cytokine production in aging. *J Biol Chem*, 273, 32833–32841.

Qin, B., Anderson, R. A., Adeli, K. (2008). TNF-α directly stimulates the overproduction of hepatic apolipoprotein B100-containing VLDL via impairment of hepatic insulin signalling. *Am J Physiol Gastrointest Liver Physiol*, 294(5), G1120–G1129.

Qu, Z., Qing, G., Rabson, A., Xiao, G. (2004). Tax deregulation of NF-κB2 p100 processing involves both β-TrCP-dependent and independent mechanisms. *J Biol Chem*, 279, 44563–44572.

Radonić, A., Thulke, S., Bae, H. G., Müller, M. A., Siegert, W., Nitsche, A. (2005). Reference gene selection for quantitative Real-Time PCR analysis in virus infected cells: SARS corona virus, Yellow fever virus, Human Herpesvirus-6, Camelpox virus, and Cytomegalovirus infections. *Virol J*, 2, 7.

Rakhshandehroo, M., Knoch, B., Müller, M., Kersten, S. (2010). Peroxisome proliferator-activated receptor alpha target genes. *PPAR Res*, 2010, 612089.

Rakoff-Nahoum, S. (2006). Why cancer and inflammation? *Yale J Biol Med*, 79(3–4), 123–130.

Rash, J. M., Tothblat, G. H., Sparks, C. E. (1981). Lipoprotein apolipoprotein synthesis by human hepatoma cells in culture. *Biochem Biophys Acta*, 666, 294–298.

Reber, L., Vermeulen, L., Haegeman, G., Frossard, N. (2009). Ser276 phosphorylation of NF-κB p65 by MSK1 controls SCF expression in inflammation. *PLos ONE*, 4(2), e4393.

Reddy, J. K., Hashimoto, T. (2001). Peroxisomal beta-oxidation and PPARα: An adaptive metabolic system. *Annu Rev Nutr*, 21, 193–230.

Reddy, J. K., Rao, S. (2006). Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol*, 290(5), G852–G858.

Reddy, S. A., Huang, J. H., Liao, W. S. (2000). Phosphatidylinositol 3-kinase as a mediator of TNF-induced NF-kappaB activation. *J Immunol*, 164, 1355–1363.

Ricote, M., Glass, C. K. (2007). PPARs and molecular mechanisms of transrepression. *Biochim Biophys Acta*, 1771(8), 926–935.

Roglans, N., Bellido, A., Rodríguez, C., Cabrero, A., Novell, F., Ros, E., Zambón, D., Laguna, J. C. (2002). Fibrate treatment does not modify the expression of acyl coenzyme A oxidase in human liver. *Clin Pharmacol Ther*, 72(6), 692–701.

Rosenfeld, M., Prichard, L., Shiojiri, N., Fausto, N. (2000). Lack of signaling through TNFR-1 rescues the embryonic lethal phenotype of RelA knockout mice. *Am J Pathol*, 156, 997–1007.

Rothe, M., Wong, S. C., Henzel, W. J., Goeddel, D. V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa TNFR. *Cell*, 78, 681–692.

Rothgiesser, K. M., Fey, M., Hottiger, M. O. (2010). Acetylation of p65 at lysine 314 is important for late NF-κB-depedent gene expression. *BMC Genomics*, 11, 22.

Ruan, H., Pownall, H. J., Lodish, H. F. (2003). Troglitazone antagonises TNF-α-induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NF-κB. *J Biol Chem*, 278(30), 28181–28192.

Rubin, D., Claas, S., Pfeuffer, M., Nothnagel. M., Foelsch, U. R., Schrezenmeir, J. (2008). s-ICAM-1 and s-VCAM-1 in healthy men are strongly associated with traits of the metabolic syndrome, becoming evident in the postprandial response to a lipid-rich meal. *Lipids Health Dis*, 7, 32.

Rubins, H. B., Robins, S. J., Collins, D., Nelson, D. B., Elam, M. B., Schaefer, E. J., Faas, F. H., Anderson, J. W. (2002). Diabetes, plasma insulin, and cardiovascular disease: subgroup analysis from the Department of Veterans Affairs high-density lipoprotein intervention trial (VA-HIT). *Arch Intern Med*, 162(22), 2597–2604.

Saad, B., Dakwar, S., Said, O., Abu-Hijleh, G., Battah, F. A., Kmeel, A., Aziazeh, H. (2005). Evaluation of medicinal plant hepatotoxicity in co-cultures of hepatocytes and monocytes. *Med*, 3(1), 93–98.

Saas, G., Shembade, N. D., Haimerl, F., Lamoureux, N., Hashemolhosseini, S., Tannapfel, A., Tiegs, G. (2007). TNF pretreatment interferes with mitochondria apoptosis in the mouse liver by A20-mediated down-regulation of Bax. *J Immunol*, 179, 7042–7049.

Saas, G., Shembade, N. D., Tiegs, G. (2005). TNF-α-TNF receptor 1-inducible cytoprotective proteins in the mouse liver: relevance of suppressors of cytokine signalling. *Biochem J*, 385, 537–544.

Saccani, S., Pantano, S., Natoli, G. (2003). Modulation of NF-κB activity by exchange of dimmers. *Mol Cell*, 11(6), 1563–1574.

Sakurai, H., Suzuki, S., Kawasaki, N., Nakano, H., Okazaki, T., Chino, A., Doi, T., Saiki, I. (2003). TNF-α-induced IKK phosphorylation of NF-κB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signalling pathway. *J Biol Chem*, 278, 36916–36923.

Sampath, H., Ntambi, J. (2004). Polyunsaturated fatty acid regulation of gene expression. *Nutr Rev*, 62, 333–339.

Sanderson, L. M., de Groot, P. J., Hooiveld, G. J., Koppen, A., Kalkhoven, E., Muller, M., Kersten, S. (2008). Effect of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics. *PLoS ONE*, 3, e1681.

Sasaki, M., Jordan, P., Welbourne, T., Minagar, A., Joh, T., Itoh, M., Elrod, J. W., Alexander, J. S. (2005a). Troglitazone, a PPARγ activator prevents endothelial cell adhesion molecule expression and lymphocyte adhesion mediated by TNF-α. *BMC Physiol*, 5, 3.

Sasaki, C. Y., Barberi, T. J., Ghosh, P., Longo, D. L. (2005b). Phosphorylation of RelA/p65 on serine 536 defines an IκBα-independent NF-κB pathway. *J Biol Chem*, 280, 34538–34547.

Satou, R., Miyata, K., Katsurada, A., Navar, L. G., Kobori, H. (2010). TNF-α suppresses angiotensinogen expression through formation of a p50/p50 homodimer in human renal proximal tubular cells. *Am J Physiol Cell Physiol*, 299(4), C750–C759.

Sawanobori, M., Yamaguchi, S., Hasegawa, M., Inoue, M., Suzuki, K., Kamiyama, R., Hirokawa, K., Kitagawa, M. (2003). Expression of TNF receptors and related signaling molecules in the bone marrow from patients with myelodysplastic syndromes. *Leukemia Res*, 27, 583–591.

Schmitz, M. L., Baeuerle, P. A. (1991). The p65 subunit is responsible for the strong transcription activating potential of NF-κB. *EMBO J*, 10, 3805–3817.

Schmuth, M., Haqq, C., Cairns, W., Holder, J., Dorsam, S., Chang, S., Lau, P., Fowler, A., Chuang, G., Moser, A., Brown, B., Mao-Qiang, M., Uchida, Y., Schoonjans, K., Auwerx, J., Chambon, P., Willson, T., Elias, P., Feingold, K. (2004). PPARβ/δ stimulates differentiation and lipid accumulation in keratinocytes. *J Invest Dermatol*, 122, 971–983.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., Staels, B., Auwerx, J. (1996). PPARα and γ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J*, 15(19), 5336–5348.

Schreiber, A. J., Simon, F. R. (1983). Estrogen-induced cholestasis: clues to pathogenesis and treatment. *Hepatology*, 3(4), 607–613.

Schwabe, R. F., Brenner, D. A. (2006). Mechanisms of liver injury. I. TNF-α-induced liver injury: role of IKK, JNK, and ROS pathways. *Am J Physiol Gastrointest Liver Physiol*, 290(4), G583–G589.

Schwabe, R. F., Sakurai, H. (2005). IKKβ phosphorylates p65 at S468 in transactivation domain 2. *FASEB J*, 19, 1758–1760.

Seidel, P., Merfort, I., Hughes, M., Oliver, B. G. G., Tamm, M., Roth, M. (2009). Dimethylfumate inhibits NF-κB function at multiple levels to limit airway smooth muscle cell cytokine secretion. *Am J Physiol Lung Physiol*, 297(2), L326–L339.

Selvey, S., Thompson, E. W., Matthaei, K., Lea, R. A., Irving, M. G., Griffiths, L. R. (2001). Beta-actin – an unsuitable internal control for RT-PCR. *Mol Cell Probes*, 15(5), 307–311.

Senftleben, U., Cao, Y., Xiao, G., Greten, F. R., Krähn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S. C., Karin, M. (2001). Activation by IKKα of a second, evolutionary conserved, NF-κB signalling pathway. *Science*, 293(5534), 1495–1499.

Sertznig, P., Seifert, M., Tilgen, W., Reichrath, J. (2007). Present Concepts and Future Outlook: Function of Peroxisome Proliferator-Activated Receptors (PPARs) for Pathogenesis, Progression, and Therapy of Cancer. *J Cell Physiol*, 212, 1–12.

Sethi, G., Sung, B., Aggarwal, B. B. (2008). TNF: A master switch for inflammation to cancer. *FBS*, 5094–5107.

Seubwai, W., Vaeteewoottacharn, K., Hiyoshi, M., Suzu, S., Puapairoj, A., Wongkham, C., Okada, S., Wongkham, S. (2010). Cepharanthine exerts antitumor activity on cholangiocarcinoma by inhibiting NF-κB. *Cancer Sci*, 101(7), 1590–1595.

Shah, A., Rader, D. J., Millar, J. S. (2010). The effect of PPARα agonism on apolipoprotein metabolism in humans. *Atherosclerosis*, 210(1), 35–40.

Shalev, A., Siegrist-Kaiser, C. A., Yen, P. M., Wahli, W., Burger, A. G., Chin, W. W., Meier, C. A. (1996). The PPARα is a phosphoprotein: regulation by insulin. *Endocrinol*, 137(10), 4499–4502.

Shearer, B. G., Hoekstra, W. J. (2003). Recent advances in peroxisome proliferator-activated receptor science. *Curr Med Chem*, 10, 267–280.

Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., Collins, T. (1999). Transriptional activation by NF-κB requires multiple coactivators. *Mol Cell Biol*, 19, 6367–6378.

Sher, T., Yi, H. F., McBride, O. W., Gonzalez, F. J. (1993). cDNA cloning, chromosomal mapping, and functional characterization of the human PPAR. *Biochem*, 32, 5598–5604.

Shi, Q. Y., Lin, Y. G., Zhou, X., Lin, Y. Q., Yan, S. (2010). Interaction among peroxisome proliferators-activated receptor alpha, cytochrome P450 oxysterol 7α-hydroxylase and estrogen receptor and its association with intrahepatic cholestasis in pregnant rats. *Zhonghua Fu Chan Ke Za Zhi*, 45(6), 440–444.

Shih, V. F. S., Tsui, R., Caldwell, A., Hoffmann, A. (2011). A single NF-κB system for both canonical and non-canonical signalling. *Cell Res*, 21, 86–102.

Siggs, O. M., Berger, M., Krebs, P., Arnold, C. N., Eidenschenk, C., Huber, C., Pirie, E., Smart, N. G., Khovananth, K., Xia, Y., McInerney, G., Hedestam, G. B. K., Nemazee, D., Beutler, B. (2010). A mutation of *Ikbkg* causes immune deficiency without impairing degradation of IκBα. *PNAS*, 107(7), 3046–3051.

Silver, N., Best, S., Jiang, J., Thein, S. L. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using Real-Time PCR. *BMC Mol Biol*, 7, 33.

Silverman, N., Maniatis, T. (2001). NF-κB signalling pathways in mammalian and insect innate immunity. *Genes Dev*, 15, 2321–2342.

Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H., Stark, G. R. (2002). Distinct roles of the IκB kinase α and β-subunits in liberating NF-κB from IκB and in phosphorylating the p65 subunit of NF-κB. *J Biol Chem*, 277(6), 3863–3869.

Slim, A., Castillo-Rojas, L., Hulten, E., Slim, J. N., Moore, D. P., Villines, T. C. (2011). Rosiglitazone and fenofibrate additive effects on lipids. *Cholesterol*, 2011, 286875.

Souvannavong, V., Saidji, N., Chaby, R. (2007). Lipopolysaccharide from *Salmonella enteric* activates NF-κB through both classical and alternative pathways in primary B lymphocytes. *Infect Immun*, 75(10), 4998–5003.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., Fruchart, J. C. (1998a). Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation*, 98, 2088–2093.

Staels, B., Koenig, W., Habib, A., Merval, R., Lebret, M., Torra, I. P., Delerive, P., Fadel, A., Chinetti, G., Fruchart, J. C., Najib, J., Maclouf, J., Tedgui, A. (1998b). Activation of human aortic smooth-muscle cells is inhibited by PPARα but not by PPARγ activators. *Nature*, 393(6687), 790–793.

Steinbrecher, K. A., Wilson, W., III., Cogswell, P. C., Baldwin, A. S. (2005). Glycogen synthase kinase 3β functions to specify gene-specific, NF-*κ*B dependent transcription. *Mol Cell Biol*, 25(19), 8444–8455.

Sterchele, P. F., Sun, H., Peterson, R. E., Vanden Heuvel, J. P. (1996). Regulation of PPARα mRNA in rat liver. *Arch Biochem Biophys*, 326(2), 281–289.

Stienstra, R., Duval, C., Muller, M., Kersten, S. (2007). PPARs, obesity, and inflammation. *PPAR Res*.

Stienstra, R., Saudale, F., Duval, C., Keshtkar, S., Groener, J. E., van Rooijen, N., Staels, B., Kersten, S., Müller, M. (2010). Kupffer cells promote hepatic steatosis via IL-1β-dependent suppression of PPARα activity. *Hepatology*, 51(2), 511–522.

Stonans, I., Stonane, E., Wiederhold, M. (1999). HepG2 human hepatoma cells express multiple cytokine genes. *Cytokine*, 112, 151–156.

Stott, W. T., Yano, B. L., Williams, D. M., Barnard, S. D., Hannah, M. A., Cieszlak, F. S., Herman, J. R. (1995). Species-dependent induction of peroxisome proliferation by haloxyfop, an aryloxyphenoxy herbicide. *Fundam Appl Toxicol*, 28, 71–79.

Sturzenbaum, S.R., Kille, P. (2001). Control genes in quantitative molecular biological techniques: The variability of invariance. *Comp Biochem Physiol Part B Biochem Mol Biol*, 130, 281–289.

Sun, S. C. (2011). Non-canonical NF-*κ*B signaling pathway. *Cell Res*, 21, 71–85.

Sun, X., Wang, X., Chen, T., Li, T., Cao, K., Lu, A., Chen, Y., Sun, D., Luo, J., Fan, J., Young, W., Ren, Y. (2010). Myelin activates FAK/Akt/NF-*κ*B pathways and provokes CR3-dependent inflammatory response in murine system. *PLoS One*, 5(2), e9380.

Szabo, G., Dolganiuc, A., Mandrekar, P. (2006). Pattern recognition receptors: A contemporary view on liver diseases. *Hepatology*, 44, 287–298.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*, 100, 655.

Tacer, K. F., Kuzman, D., Seliškar, M., Pompon, D., Rozman, D. (2007). TNF-α interferes with lipid homeostasis and activates acute and proatherogenic processes. *Physiol Genomics*, 31(2), 216–227.

Tan, N. S., Michalik, L., Desvergne, B., Wahli, W. (2005). Multiple expression control mechanisms of PPARs and their target genes. *J Steroid Biochem and Mol Bio*, 93(2–5), 99–105.

Tang, W., Eggertsen, G., Chiang, J. Y., Norlin, M. (2006). Estrogen-mediated regulation of CYP7B1: A possible role for controlling DHEA levels in human tissues. *J Steroid Biochem Mol Biol*, 100(1–3), 42–51.

Tekkatte, C., Gunasingh, G. P., Cherian, K. M., Sankaranarayanan, K. (2011). "Humanised" stem cell culture techniques: the animal serum controversy. *Stem Cells Int*, 2011, 504723.

Tergaonkar, V. (2006). NF-κB pathway: A good signalling paradigm and therapeutic target. *Int J Biochem Cell Biol*, 38, 1647–1653.

Teshima, S., Nakanishi, H., Nishizawa, M., Kitagawa, K., Kaibori, M., Yamada, M., Habara, K., Kwon, A. H., Kamiyama, Y., Ito, S., Okumura, T. (2004). Up-regulation of IL-1 receptor through PI3K/Akt is essential for the induction of iNOS gene expression in hepatocytes. *J Hepatol*. 40, 616–623.

Tham, D. M., Martin-McNulty, B., Wang, Y. X., Wilson, D. W., Vergona, R., Sullivan, M. E., Dole, W., Rutledge, J. C. (2002). Angiotensin II is associated with activation of NF-κB-mediated genes and down-regulation of PPARs. *Physiol Genomics*, 11, 21–30.

Thellin, O., Zorzi, W., Lakaye, B., De, B. B., Coumans, B., Hennen, G., Grisar, T., Igout, A., Heinen, E. (1999). Housekeeping genes as internal standards: Use and limits. *J Biotechnol*, 75, 291–295.

Tian, B., Brasier, A. R. (2003). Identification of a NF-κB-dependent gene network. *Recent Progress in Hormone Res*, 58, 95–130.

Tsou, H. K., Su, C. M., Chen, H. T., Hsieh, M. H., Lin, C. J., Lu, D. Y., Tang, C. H., Chen, Y. H. (2010). Integrin-linked kinase is involved in TNF-α-induced inducible nitric-oxide synthase expression in myoblasts. *J Cell Biochem*, 109(6), 1244–1253.

Tulac, S., Dosiou, C., Suchanek, E., Giudice, L. C. (2004). Silencing lamin A/C in human endometrial stromal cells: A model to investigate endometrial gene function and regulation. *Mol Hum Reprod*, 10(10), 705–711.

Udalova, I. A., Richardson, A., Denys, A., Smith, C., Ackerman, H., Foxwell, B., Kwiatkowski, D. (2000). Functional consequences of a polymorphism affecting NF-κB p50-p50 binding to the TNF promoter region. *Mol Cell Biol*, 20(24), 9113–9119.

Uings, I., Puxeddu, I., Temkin, V., Smith, S. J., Fattah, D., Ray, K. P., Levi-Schaffer, F. (2005). Effects of dexamethasome on TNF-α-induced release of cytokines from purified human blood eosinophils. *Clinical and Molecular Allergy*, 3, 5.

van Antwerp D. J., Martin, S. J., Verma, I. M., Green, D. R. (1998). Inhibition of TNF-induced apoptosis by NF-κB. *Trends Cell Biol*, 8, 107–111.

van der Lee, K. A., Willemsen, P. H., Samec, S., Seydoux, J., Dulloo, A. G., Pelsers, M. M., Glatz, J. F., Van der Vusse, G. J., Van Bilsen, M. (2001). Fasting-induced changes in the expression of genes controlling substrate metabolism in the rat heart. *J Lipid Res*, 42(11), 1752–1758.

Vanden Berghe, W., Vermeulen, L., Delerive, P., De Bosscher, K., Staels, B., Haegeman, G. (2003). A paradigm for gene regulation: inflammation, NF-κB and PPAR. *Adv Exp Med Biol*, 544, 181–196.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002). Accurate normalisation of Real-Time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genomic Biol*, 3, 1-11.

Vega, R. B., Huss, J. M., Kelly, D. P. (2000). The coactivator PGC-1 cooperates with PPARα in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol*, 20, 1868–1876.

Vu-Dac, N., Schoonjans, K., Kosykh, V., Dallongeville, J., Fruchart, J. C., Staels, B., Auwerx, J. (1995). Fibrates increase human ApoA-II expression through activation of the PPAR. *J Clin Invest*, 96(2), 741–750.

Wajant, H., Pfizenmaier, K., Scheurich, P. (2003). Tumor necrosis factor signalling. *Cell Death Differ*, 10, 45–65.

Wallach, D., Holtmann, H., Engelmann, H., Nophar, Y. (1988). Sensitisation and desensitisation to lethal effects of TNF and IL-1. *J Immunol*, 140, 2994–2999.

Wang, D., Westerheide, S. D., Hanson, J. L., Baldwin, A. S. Jr. (2000). TNF-α-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. *J Biol Chem*, 275, 32592–32597.

Wang, G. R., Guo, X. X., Floros, J. (2005). Differences in the translation efficiency and mRNA stability mediated by 5'-UTR splice variants of human SP-A1 and SP-A2 genes. *Am J Physiol Lung Cell Mol Physiol*, 289 (3), L497–L508.

Wang, S. R., Pessah, M., Infante, J., Catala, D., Salvat, C., Infante, R. (1988). Lipid and lipoprotein metabolism in HepG2 cells. *Biochem Biophys Acta*, 961, 351–363.

Wang, W. L., Lee, Y. C., Yang, W. M., Chang, W. C., Wang, J. M. (2008). Sumoylation of LAP1 is involved in the HDAC4-mediated repression of *COX-2* transcription. *Nucl Acids Res*, 36(19), 6066–6079.

Werman, A., Hollenberg, A., Solanes, G., Bjorbaek, C., Vidal-Puig, A. J., Flier, J. S. (1997). Ligand-independent activation domain in the N terminus of PPAR-γ. Differential activity of PPAR-γ 1 and 2 isoforms and influence of insulin. *J Biol Chem*, 272, 20230–20235.

Westergaard, M., Henningsen, J., Johansen, C., Rasmussen, S., Svendsen, M. L., Jensen, U. B., Schrøder, H. D., Staels, B., Iversan, L., Bolund, L., Kragballe, K., Kristiansen, K. (2003). Expression and localisation of PPARs and NF-κB in normal and lesional psoriatic skin. *J Invest Dermatol*, 121(5), 1104–1107.

White, I. R., Man, W. J., Bryant, D., Bugelski, P., Camilleri, P., Cutler, P., Hayes, W., Holbrook, J. D., Kramer, K., Lord, P. G., Wood, J. (2003). Protein expression changes in the Sprague Dawley rat liver proteome following administration of PPARα and γ ligands. *Proteomics*, 3(4), 505–512.

Whitsett, J. A., Clark, J. C., Wispe, J. R., Pryhuber, G. S. (1992). Effects of TNF-α and phorbol ester on human surfactant protein and MnSOD gene transcription *in vitro*. *Am J Physiol Lung Cell Mol Physiol*, 262(6), L688–L693.

Willson, T. M., Brown, P. J., Sternbach, D. D., Henke, B. R. (2000). The PPARs: From Orphan Receptors to Drug Discovery. *J Med Chem*, 43(4), 527–550.

Willson, T. M., Lambert, M. H., Kliewer, S. A. (2001). Peroxisome proliferator-activated receptor gamma and metabolic disease. *Annu Rev Biochem*, 70, 341–367.

Wispé, J. R., Clark, J. C., Warner, B. B., Fajardo, D., Hull, W. E., Holtzman, R. B., Whitsett, J. A. (1990). TNF-α inhibits expression of pulmonary surfactant protein. *J Clin Invest*, 86, 1954–1960.

Wittwer, T., Schmitz, M. L. (2008). NIK and Cot cooperate to trigger NF-κB p65 phosphorylation. *Biochem Biophys Res Commun*, 371, 294–297.

Wu, Z. R., Wu, L. R., Weng, D. S., Xu, D. Z., Geng, J., Zhao, F. (2009). Reduced expression of lamin A/C correlates with poor histological differentiation and prognosis in primary gastric carcinoma. *J Exp Clin Cancer Res*, 28, 8.

Wu, Z., Chiang, J. Y. (2001). Transcriptional regulation of human oxysterol 7 alpha-hydroxylase gene (CYP7B1) by Sp1. *Gene*, 272(1–2), 191–197.

Wullaert, A., Loo, G., Heyninck, K., Beyaert, R. (2007). Hepatic TNF signalling and NF-κB: Effects on liver homeostasis and beyond. *Endocr Rev*, 28(4), 365–386.

Wunderlich, F. T., Luedde, T., Singer, S., Schmidt-Supprian, M., Baumgarti, J., Schimacher, P., Pasparakis, M., Brüning, J. C. (2008). Hepatic NF-κB essential modulator deficiency prevents obesity-induced insulin resistance but synergises with high-fat feeding in tumorigenesis. *Proc Natl Acad Sci USA*, 105(4), 1297–1302.

Xiao, G., Fong, A., Sun, S. (2004). Induction of p100 processing by NF-κB-inducing kinase involves docking IκB kinase α (IKKα) to p100 and IKKα-mediated phosphorylation. *J Biol Chem*, 279(29), 30099–30105.

Xiao, G., Harhaj, E. W., and Sun, S. C. (2001). NF-κB-inducing kinase regulates the processing of NF-κB2p100. *Mol Cell*, 7(2), 401–409.

Xing, D., Gong, K., Feng, W., Nozell, S. E., Chen, Y. F., Chatham, J. C., Oparil, S. (2011). *O*-GlcNAc modification of NF-κB p65 inhibits TNF-α-induced inflammatory mediator expression in rat aortic smooth muscle cells. *PLoS ONE*, 6(8), e24021.

Xu, H. E, Lambert, M. H., Montana, V. G., Parks, D. J., Blanchard, S. G., Brown, P. J., Sternbach, D. D., Lehmann, J. M., Wisely, W. G., Willson, T. M., Kliewer, S. A., Milburn, M. V. (1999). Molecular recognition of fatty acids by PPARs. *Mol Cell*, 3, 397–403.

Xu, H. E., Lambert, M. H., Montana, V. G., Plunket, K. D., Moore, L. B., Collins, J. L., Oplinger, J. A., Kliewer, S. A., Gampe, R. T. Jr., McKee, D. D., Moore, J. T., Willson, T. M. (2001). Structural determinants of ligand binding selectivity between the PPARs. *Proc Natl Acad Sci USA*, 98, 13919–13924.

Xu, N., Ahrén, B., Jiang, J., Nilsson-Ehle, P. (2006). Down-regulation of apolipoprotein M expression is mediated by phosphatidylinositol 3-kinase in HepG2 cells. *Biochim Biophys Acta*, 1761(2), 256–260.

Xu, Y., Bialik, S., Jones, B. E., Iimuro, Y., Kitsis, R. N., Srinivasan, A., Brenner, D. A., Czaja, M. J. (1998). NF-κB inactivation converts a hepatocyte cell line TNF-α response from proliferation to apoptosis. *Am J Physiol*, 275, C1058–C1066.

Yan, L., Tao, M. (2007). Effects of serum starvation and contact inhibitor on the cell cycle G0 synchronisation of the human embryonic lung fibroblast. *Wei Sheng Yan Jiu*, 36(3), 275–278.

Yang, F., Tang, E., Guan, K., Wang, C. Y. (2003). IKKβ plays an essential role in the phosphorylation of RelA/p65 on serine 536 induced by lipopolysaccharide. *J Immunol*, 170(11), 5630–5635.

Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., Su, B. (2001). The essential role of MEKK3 in TNF-induced NF-*κ*B activation. *Nat Immunol*, 2, 620–624.

Yang, Y., Gocke, A. R., Lovett-Racke, A., Drew, P. D., Racke, M. K. (2008). PPARα regulation of the immune response and autoimmune encephalomyelitis. *PPAR Res*.

Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M., Lazar, M. A. (1995). Differential activation of PPARs by eicosanoids. *J Biol Chem*, 270(41), 23975–23983.

Yu, S., Reddy, J. K. (2007). Transcription coactivators for peroxisome proliferator-activated receptors. *Biochim Biophys Acta*, 1771(8), 936–951.

Yuan, J. S., Reed, A., Chen, F., Stewart, C. N. (2006). Statistical analysis of Real-Time PCR data. *BMC Bioinformatics*, 7, 85.

Zambon, A., Gervois, P., Pauletto, P., Fruchart, J. C., Staels, B. (2006). Modulation of hepatic inflammatory risk markers of cardiovascular diseases by PPARα activators. *Arterioscler Thromb Vasc Biol*, 26(5), 977–986.

Zhong, H., May, M. J., Jimi, E., Ghosh, S. (2002). The phoshorylation status of nuclear NF-*κ*B determines its association with CBP/p300 or HDAC-1. *Mol Cell*, 9(3), 625–636.

Zhong, H., Simons, J. W. (1999). Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem Biophys Res Commun*, 259(3), 523–526.

Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., Ghosh, S. (1997). The transcriptional activity of NF-*κ*B is regulated by the I*κ*B-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell*, 89, 413–424.

Zhou, Z., Gengaro, P., Wang, W., Wang X. Q., Li, C., Faubel, S., Rivard, S., Schrier, R. W. (2008). Role of NF-*κ*B and PI3-kinase/Akt in TNF-α-induced cytotoxicity in microvascular endothelial cells. *Am J Physiol Renal Physiol*, 295(4), F932–F941.

Zierath, J. R., Ryder, J. W., Doebber, T., Woods, J., Wu, M., Ventre, J., Li, Z., McCrary, C., Berger, J., Zhang, B., Moller, D. E. (1998). Role of skeletal muscle in thiazolidinedione insulin sensitizer (PPARγ agonist) action. *Endocrinology*, 139(12), 5034–5041.

Zoete, V., Grosdidier, A., Michielin, O. (2007). PPAR structures: ligand specificity, molecular switch and interactions with regulators. *Biochim Biophys Acta-Mol Cell Biol Lipids*, 1771(8), 915–925.

Zomer, A. W., van Der Burg, B., Jansen, G. A., Wanders, R. J., Poll-The, B. T., van Der Saag, P. T. (2000) Pristanic acid and phytanic acid: Naturally occurring ligands for the nuclear receptor PPARα. *J. Lipid Res*, 41, 1801–1807.

APPENDICES

Appendix A

Representative data for the quality and quantity of the extracted total cellular RNA isolated from HepG2 cells by using NanoDrop 1000 Spectrophotometer. The A_{260}/A_{280} ratio is used to assess RNA purity. The total RNA used in this study had an A_{260}/A_{280} ratio of 1.8 – 2.1 which is indicative of highly purified RNA.

| RNA sample	A_{260}	A_{280}	A_{260}/A_{280} Ratio	A_{260}/A_{230} Ratio	Concentration (ng/μl)
1	37.031	17.985	2.06	2.22	1851.5
2	52.291	25.267	2.07	2.18	2614.5
3	37.046	17.722	2.09	2.23	1852.3
4	45.622	22.674	2.01	2.14	2281.1
5	49.587	23.841	2.08	1.93	2479.3

Appendix B

Universal Pfaffl formula used to calculate the relative expression folds for Real-Time RT-PCR samples.

$$\text{Relative expression ratio} \ = \ \frac{(E_{target})^{\Delta CT \ target \ (control \ - \ treated)}}{(E_{reference})^{\Delta CT \ reference \ (control \ - \ treated)}}$$

Appendix C

Effect of a panel of inhibitors on TNF-α inhibitory action on PPARα gene expression in HepG2 cells. The results were normalised to β-actin expression. Treatment with 50μM of Wedelolactone, which is the NF-κB pathway inhibitor, can effectively induce PPARα expression in TNF-α-stimulated cells if compared among all the kinase inhibitors that have been used (Results shown here were obtained from predecessors).
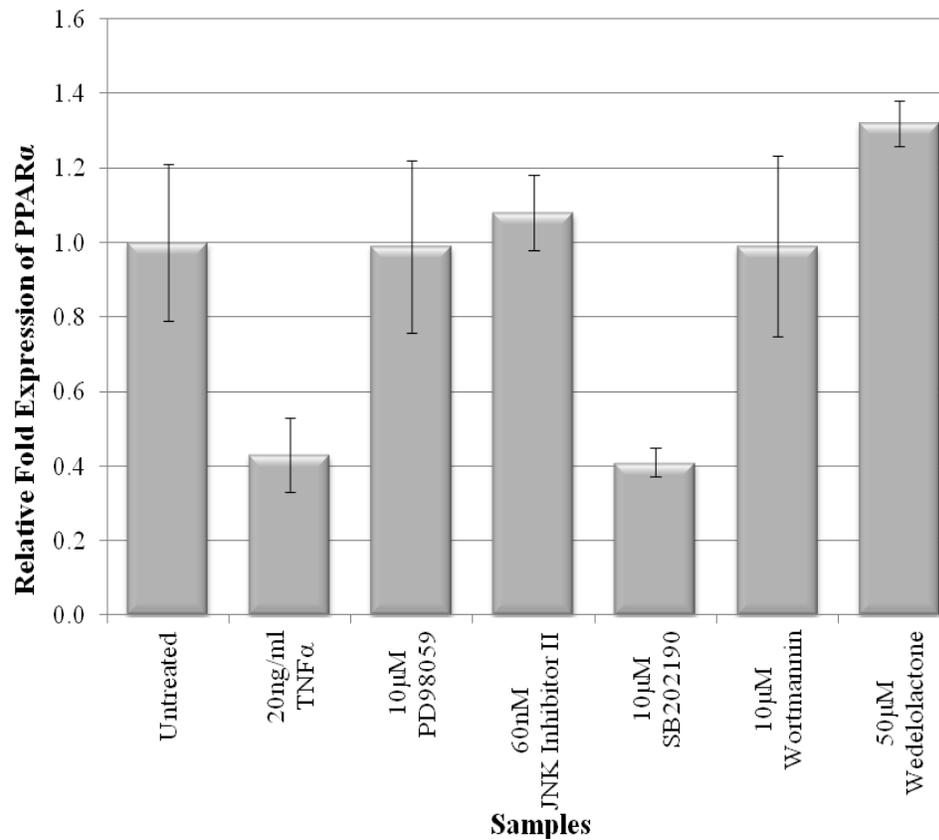
Effect of vehicle-treated cells on the PPARα expression. The results were normalised to β-actin expression. There is no effect in vehicle-treated cells, as compared with the untreated cells.