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

By
LIM WYI SIAN

A thesis submitted to the Department of Biomedical Science
Faculty of Science
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In partial fulfillment of the requirements for the degree of
Master of Science
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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

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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.

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Faculty of Science
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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: ________________
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<td>A&lt;sub&gt;230&lt;/sub&gt;</td>
<td>Absorbance at 230 nm wavelength</td>
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<td>A&lt;sub&gt;260&lt;/sub&gt;</td>
<td>Absorbance at 260 nm wavelength</td>
</tr>
<tr>
<td>A&lt;sub&gt;280&lt;/sub&gt;</td>
<td>Absorbance at 280 nm wavelength</td>
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<tr>
<td>AF</td>
<td>Activation function</td>
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<td>Alanine</td>
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<td>Apolipoprotein</td>
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<td>ARP</td>
<td>ApoAI regulatory protein</td>
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<td>bp</td>
<td>Base pair</td>
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<td>C/EBP</td>
<td>CCAAT-enhancer-binding protein</td>
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<td>CREB</td>
<td>cyclic AMP response element-binding protein</td>
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<td>CBP</td>
<td>CREB-binding protein</td>
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<td>Cyclooxygenase-2</td>
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<td>C-reactive protein</td>
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<td>DNA binding domain</td>
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<td>DMSO</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Dnmt3</td>
<td>DNA methyltransferase</td>
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<td>dNTPs</td>
<td>Deoxyribonucleoside triphosphates</td>
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<td>DR</td>
<td>Direct repeat</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>FADD</td>
<td>FAS-associated death domain</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>GABP</td>
<td>GA-binding protein</td>
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<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
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<td>HAT</td>
<td>histone acetyl transferase</td>
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<td>HDAC</td>
<td>histone deacetylase</td>
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<td>HDL</td>
<td>High-density lipoprotein</td>
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<td>HepG2</td>
<td>Hepatocellular carcinoma</td>
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<td>HNF4</td>
<td>Hepatic nuclear factor 4</td>
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<td>IKK</td>
<td>IκB kinase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IκB</td>
<td>Inhibitory protein of NF-κB</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>kb</td>
<td>Kilobase</td>
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<td>kPa</td>
<td>Kilo pascal</td>
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<td>LBD</td>
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<td>MAP3K</td>
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<td>MAPK</td>
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<td>MEF</td>
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<td>MEKK3</td>
<td>Mitogen-activated protein 3 kinase</td>
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<tr>
<td>MEM</td>
<td>Eagle’s Minimum Essential Medium</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MSK1</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>NCoR</td>
<td>Nuclear-receptor corepressor complex</td>
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<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
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<tr>
<td>NF-AT</td>
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<tr>
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</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPRE</td>
<td>Peroxisome proliferator response element</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
</tr>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>rpm</td>
<td>Revolutions per minute</td>
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<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<td>RXR</td>
<td>Retinoid X receptor</td>
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<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoid and thyroid hormone receptor</td>
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<td>SRC</td>
<td>Steroid receptor coactivator</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>TAD</td>
<td>Transcriptional activation domain</td>
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<td><em>Thermus aquaticus</em></td>
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<td>Thr</td>
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Lever 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; Hansmann 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, atherosclerosis and glucose homeostasis (Devchand et al., 1996; Staels et al., 1998; Staels 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
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 (Kliwer 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 LXXXIXXL (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 phosphorylation (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 a 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.

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

NF-κB is a group of structurally related transcriptional proteins that form homodimers or heterodimers composed of various combinations of members of the mammalian NF-κB transcription factor family, which are NF-κB1 (p50, its precursor p105), NF-κB2 (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-κB protein genes did indeed reveal that different NF-κB-regulated genes have distinct requirements for NF-κB proteins (Hoffmann et al., 2003). However, different forms of the NF-κB dimer exhibit distinct properties in terms of DNA binding preference selectively of interaction with IκB isoforms, and transcriptional capability. NF-κB transcriptional activity is silenced by interactions with IκB proteins through three mechanisms, by sequestrating NF-κB dimers in the cytoplasm, facilitating dissociation of DNA-bound NF-κB dimers from their DNA binding sites, and exporting NF-κB dimers from nucleus (Liu and Malik, 2006). Thus, NF-κB is present as a latent, IκB-bound complex in the cytoplasm in most cells.

There are two signalling pathways leading to the activation of NF-κB 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-κB signalling pathways. Although they are often activated concurrently, the two NF-κB 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 (IKKa 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-κB signalling pathway.

PPARα interference with the NF-κB 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 IκBα, 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).
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) CO₂ at 37°C. The cell culture medium in the tissue culture flask (25 cm²) was replaced every 3 days.
Table 3.1  Materials used in the project and their suppliers.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol blue, Ethylenediaminetetraacetic acid (EDTA), Isopropanol,</td>
<td>Amresco</td>
</tr>
<tr>
<td>Acetone, Ammonium persulphate (APS), Acrylamide-bisacrylamide™ 37.5:1</td>
<td></td>
</tr>
<tr>
<td>(w/v) solution, Phosphate-buffered saline (PBS), Tween®, 20, Actinomycin</td>
<td></td>
</tr>
<tr>
<td>D, Guanidine hydrochloride</td>
<td></td>
</tr>
<tr>
<td>QuantiFast™ SYBR® Green RT-PCR Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Ethanol</td>
<td>HmbG Chemicals</td>
</tr>
<tr>
<td>Sodium chloride (NaCl), Bovine serum albumin (BSA)-fraction V, Methanol,</td>
<td>Merck</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td></td>
</tr>
<tr>
<td>Penicillin/Streptomycin, Trypsin-EDTA, 1-bromo-3-chloropropane (BCP)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Minimum essential medium (MEM), Foetal bovine serum (FBS), Sodium pyruvate,</td>
<td>Gibco</td>
</tr>
<tr>
<td>Non-essential amino acids</td>
<td></td>
</tr>
<tr>
<td>Prestained protein marker (broad range)</td>
<td>Biolabs</td>
</tr>
<tr>
<td>HepG2 cell line</td>
<td>American Type Culture</td>
</tr>
<tr>
<td>TRI- Reagent® LS</td>
<td>Molecular Research Centre</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS), Glycerol</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Tris, Glycine</td>
<td>Promega</td>
</tr>
<tr>
<td>NE-PER® Nuclear and Cytoplasmic Extraction Reagents</td>
<td>Pierce</td>
</tr>
<tr>
<td>Reverse primer, Forward primer</td>
<td>Research Biolabs</td>
</tr>
<tr>
<td>Protease inhibitor cocktail Set I, IKK inhibitor II (Wedelolactone), NF-κB</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>activation inhibitor IV, IKK-2 inhibitor (SC-514), Tumor necrosis factor-alpha (TNF-α)</td>
<td></td>
</tr>
<tr>
<td>Cell scrapers, Tissue culture flasks (25 cm² and 75 cm²), Cryo-vials</td>
<td>Techno Plastic Products (TPP)</td>
</tr>
</tbody>
</table>
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) CO₂ 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 cm² 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) CO₂).
3.2.3 Cells storage

When the cells in 25 cm² 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 cm² 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) CO₂.

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) CO₂.

3.6 Isolation of total cellular RNA

HepG2 cells were cultured and maintained in a 25 cm² tissue culture flask until they reached 70% confluence. Total cellular RNA was isolated from cells cultured in 25 cm² 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 ddH₂O 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™ 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™ 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™ 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 4°C.

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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Expected size/bp</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin_F</td>
<td>TCACCCTGAAGTACCACCACATC</td>
<td>489</td>
<td>Chew et al., 2006</td>
</tr>
<tr>
<td>β-actin_R</td>
<td>CCATCTTTTGCTCGAAGTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARα_F</td>
<td>CCGTTATCTGAAGAGTTCCCTG</td>
<td>440</td>
<td>Chew et al., 2006</td>
</tr>
<tr>
<td>PPARα_R</td>
<td>GTTGTGTGACATCCCCGACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoAI_F</td>
<td>CTGGCCACTGTGTACGTGGATG</td>
<td>310</td>
<td>Matsuura et al., 2007</td>
</tr>
<tr>
<td>ApoAI_R</td>
<td>TGCCGCGTAGAGCTCCATCTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoB_F</td>
<td>CTGGGAAAAACTCCCAACAGCAAG</td>
<td>414</td>
<td>Matsuura et al., 2007</td>
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<tr>
<td>ApoB_R</td>
<td>CCACATTGGTAATCCAGGATGCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3  Real-Time RT-PCR cycle and melting curve cycle (Chew et al., 2007).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>50°C</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>PCR initial activation step</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>30 sec</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Melting curve</td>
<td>95°C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>60°C – 95°C</td>
<td>10 sec</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

(increase set point temperature by 0.5°C)
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 sedimentsed at 12,000 g for 10 min at 4°C. 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 hydrochloride 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 sedimentsed 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 sedimentsed 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 -20°C until further downstream processing.

3.8.2 Isolation of nuclear and cytoplasmic proteins

Nuclear and cytoplasmic proteins were isolated from cells cultured in 25 cm² 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 Dₖ protein assay

The concentration of extracted proteins (Section 3.8.1 and Section 3.8.2) was determined by using the Bio-Rad Dₖ 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.

<table>
<thead>
<tr>
<th>Gel component</th>
<th>10% (w/v) Resolving gel</th>
<th>5% (w/v) Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE upper gel buffer</td>
<td>-</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>SDS-PAGE lower gel buffer</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>40% (w/v) acrylamide:bisacrylamide</td>
<td>2.5 ml</td>
<td>0.625 ml</td>
</tr>
<tr>
<td>(37.5:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>4.89 ml</td>
<td>3.07 ml</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>100 μl</td>
<td>50 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
<td>5 μl</td>
</tr>
</tbody>
</table>
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 ddH₂O 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 ddH₂O. 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 4°C.

### 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

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE upper gel buffer</td>
<td>0.5 M Tris, 10% (w/v) SDS, pH 6.8</td>
</tr>
<tr>
<td>SDS-PAGE lower gel buffer</td>
<td>1.5 M Tris, 10% (w/v) SDS, pH 6.8</td>
</tr>
<tr>
<td>SDS-PAGE running buffer</td>
<td>25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS</td>
</tr>
<tr>
<td>Towbin transfer buffer</td>
<td>25 mM Tris, 192 mM Glycine, 20% (v/v) methanol</td>
</tr>
<tr>
<td>SDS-PAGE gel loading buffer</td>
<td>0.12 M Tris, 25% (v/v) glycerol, 2% (w/v) SDS, 0.004% (w/v) bromophenol blue,</td>
</tr>
<tr>
<td></td>
<td>5% (v/v) 2-mercaptoethanol, pH 6.8</td>
</tr>
<tr>
<td>Tris-buffered saline-Tween 20 (TBS-T)</td>
<td>10 mM Tris, 200 mM NaCl, 0.1% (v/v) Tween 20, pH 7.4</td>
</tr>
<tr>
<td>(washing solution)</td>
<td></td>
</tr>
<tr>
<td>Blocking solution</td>
<td>1% (w/v) BSA, 10 mM Tris, 200 mM NaCl, 0.1% (v/v) Tween 20</td>
</tr>
<tr>
<td>Stripping solution</td>
<td>0.4 M Glycine, 0.2% (w/v) SDS, 2% (v/v) Tween 20, pH 2.2</td>
</tr>
</tbody>
</table>
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 ddH₂O. 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 ddH₂O 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

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


incubated in 1 ml of blocking solution containing of the secondary antibody, horseradish peroxidase (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). Prestained 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% confluence 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 $A_{230}$. 

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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™ 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.
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 misannealed 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).
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
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.

**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
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.

Briefly, 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.
After pre-incubation with 10 \( \mu \text{M} \) of Wedelolactone for 2 h, HepG2 cells were stimulated with 20 ng/ml of TNF-\( \alpha \) for 4 h (Section 3.5). PPAR\( \alpha \) 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 PPAR\( \alpha \) expression were in accordance with those obtained in Section 4.5. Compared to the controls (assigned as DMSO), TNF-\( \alpha \) 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-\( \alpha \) 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-\( \alpha \) stimulation without Wedelolactone pre-treatment.

4.8 Investigation of the mechanisms of NF-\( \kappa \)B signalling pathway

4.8.1 Activation of classical NF-\( \kappa \)B signalling pathway abrogated TNF-\( \alpha \) down-regulatory effect on PPAR\( \alpha \) expression

NF-\( \kappa \)B activation consists of two separate pathways (Bhatnagar et al., 2010). Although they are often activated concurrently, classical and alternative NF-\( \kappa \)B activation pathways have distinct regulatory functions (Nishikori, 2005). Therefore, to elucidate whether TNF-\( \alpha \) triggers the classical or alternative NF-\( \kappa \)B signalling pathway, the ability of TNF-\( \alpha \) 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-\( \alpha \) for the time indicated (Section 3.8.2 and Section 3.8.4). The \( \beta \)-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 pretreatment. 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, phosphorylation-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 Wedelolactone 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 density 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
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 density 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 density 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 immunoblotting 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
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 density 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 transcriptional 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 similarly (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 a 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 IκB proteins

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

According to Figure 4.13b, d, and f, it was demonstrated that the phosphorylation of IKKα/β (Ser180/181), IκBα (Ser32), and IκBβ (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 4.14c). 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 cytoplasmic 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.,
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 subunit (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-researchers (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
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-researchers (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.


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.

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<th>$A_{280}$</th>
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<th>$A_{260}/A_{230}$ Ratio</th>
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Appendix B

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

Relative expression ratio = \frac{(E_{\text{target}})^{ΔCT \text{ target (control – treated)}}}{(E_{\text{reference}})^{ΔCT \text{ 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).
Appendix D

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.