TITLE

The effects of Prostaglandin J_2 metabolite on the selected apoptotic signaling molecules in HeLa cells

By

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CERTIFICATE

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LIST OF ABBREVIATIONS

β-ME	β-Mercaptoethanol
15d-PGJ_2	15-deoxy- $\Delta^{12,14}$-prostaglandin J_2
AF-1	activation function-1
AF-2	activation function-2
AP	Ammonium persulfate
BSA	Bovine serum albumin
Caspase	cysteinyl-aspartic acid protease
CDK	cyclin-dependent kinase
CO_2	carbon dioxide
CoA	co-activator
CoR	co-repressor
DBD	DNA binding domain
DMEM	dulbeco modified eagle's media
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	effective concentration of 15d-PGJ_2 to cause 50% cell death
EDTA	ethylenediamine-tetra acetic acid
FasL	Fas ligand
FBS	foetal bovine serum
g	gram
kDa	kilo Dalton
KH_2PO_4	potassium dihydrogen orthophosphate
L	litre
LBD	ligand binding domain
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
Na_2HPO_4	di-sodium hydrogen orthophosphate anhydrous
Na_3VO_4	sodium orthovanadate
NaCl	sodium chloride
NaOH	sodium hydroxide
N-CoR	nuclear receptor co-repressor
NCR	National Cancer Registry
NF- κB	nuclear factor κB
NP-40	nonidet-P40
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element

RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
tBid	truncated Bid
TBS	tris-buffered saline
TEMED	N,N,N'N'-tetra-methylethylenediamine
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TZD	thiazolidinedione

LIST OF SYMBOLS

μ	micro
α	alpha
β	beta
κ	kappa
γ	gamma
δ	delta
™	trademark
°C	degree Celcius

ABSTRACT

Cancer of the cervix is one of the most rapidly increasing malignancies throughout the world and rated as the second most common cancer in Malaysian women. A ligand-dependent nuclear receptor, peroxisome proliferator-activated receptor gamma (PPARγ) has been reported to be expressed in various cancer cells including breast, prostate, colorectal and cervical cancer. Recently, down regulation of PPARγ has been reported in human cervical carcinoma. Investigations have demonstrated that activation of PPARγ is linked to growth inhibition of various cancers by induction of necrosis, apoptosis and growth arrest. This study aims to identify the effect of selective PPARγ ligand, 15-Deoxy-prostaglandin J_2 (15d-PGJ_2) on selected apoptotic molecules in the cervical cancer cell line, HeLa. HeLa cells were cultured and treated with an EC_{50} dose of 15d-PGJ_2. These cells were harvested at every sixth hour of incubation period starting from 0 – 72 hours. Western blot analysis revealed that Akt expression (anti apoptotic) was down regulated, while caspase 9 and caspase 3 (both are pro apoptotic) expression was up regulated. This finding provides significant evidence on the mechanism of apoptotic action of PPARγ ligand that is potentially useful as a chemotherapeutic agent.

ABSTRAK

Kanser serviks merupakan penyakit yang paling menular dan kanser kedua paling lazim dikalangan wanita Malaysia. Reseptor gama teraktif perangsang peroksisom (PPARγ) telah dijumpai pada pelbagai jenis sel kanser iaitu payu dara, prostat, kolorektal dan serviks. Kebelakangan ini, penurunan regulasi PPARγ telah dilaporkan pada karsinoma serviks manusia. Penyelidikan telah membuktikan bahawa pengaktifan PPARγ berkait rapat dengan perencatan tumbesaran pelbagai jenis kanser melalui nekrosis, apoptosis dan perencatan tumbesaran. Objektif kajian ialah untuk mengenal pasti kesan pemilihan ligan PPARγ, 15-Deoksi-prostagladin J_2 (15d-PGJ_2) terhadap molekul apoptosis terpilih dalam sel kanser serviks, sel HeLa. Sel HeLa dikultur dan dirawat dengan 15d-PGJ_2 pada dos EC_{50}nya. Sel-sel ini dituai setiap enam jam bermula dari 0-72 jam semasa pengeramannya. Analisis Western-blot menunjukkan bahawa pengekspresan protein Akt (anti apoptosis) mengalami penurunan regulasi. Manakala, caspase-9 dan 3 (kedua-duanya pro apoptosis) menunjukkan peningkatan dalam regulasi. Hasil penyelidikan ini memberikan bukti yang signifikan tentang mekanisma apoptosis untuk PPARγ yang berpotensi digunakan sebagai agen kemoterapi yang efektif.

1. INTRODUCTION

1.1 Cancer

A balance is normally maintained between cell birth rate and cell death rate which is strictly regulated under normal conditions, thus avoiding any abnormal tissue or organ formation (Price and Wilson, 1997). A tumor or neoplasia is observed in the form of a mass of extra tissue when cells keep dividing while new cells are not needed (Tamir, 2002).

1.1.1 Cancer of the Cervix

Cervical cancer is one of the most common cancers in women and remains a significant health care problem worldwide (Jung *et al.*, 2005). Despite significant advancements in the screening and treatment of cervical dysplasia, cervical cancer remains a threat to thousands of women annually. Cervical cancer is still a major cause of death in developing countries where it is generally considered to be the number one or two cancer-related killers of women, with nearly 500,000 new cases still being diagnosed worldwide every year (Monk, 2005).

Essentially all cancers of the cervix including non-squamous tumors (adenocarcinomas) are related to chronic infections with the human papilloma virus (HPV) (Bosch *et al.*, 1995). However, the discrepancy and high rates of HPV infection among women and low rates of cervical cancer development suggest that additional genetic events are necessary for progression to a malignant phenotype.

Indeed, the risk of progression is affected by many other factors including environmental factors and somatic genetic alterations. The identification of biomarkers associated with this process might help elucidate the molecular events and mechanisms associated with cervical carcinogenesis and to identify prognostic or predictive markers.

1.1.2 Incidence of Cervical Cancer in Malaysia

Cancer of the cervix is the second most common cancer among the women of Peninsular Malaysia following breast cancer. It constituted 12.9% of all female cancers (Figure 1.1). The National Cancer Registry (NCR), Ministry of Health, Malaysia reported 1,557 cases of cervical cancer in the year 2003. Cervical cancer incidence rate increased with age after 30 years with a peak incidence rate at ages 60-69 years, and declined thereafter. Chinese women had the highest rate of cervical cancer, followed by Indians and lastly Malays.

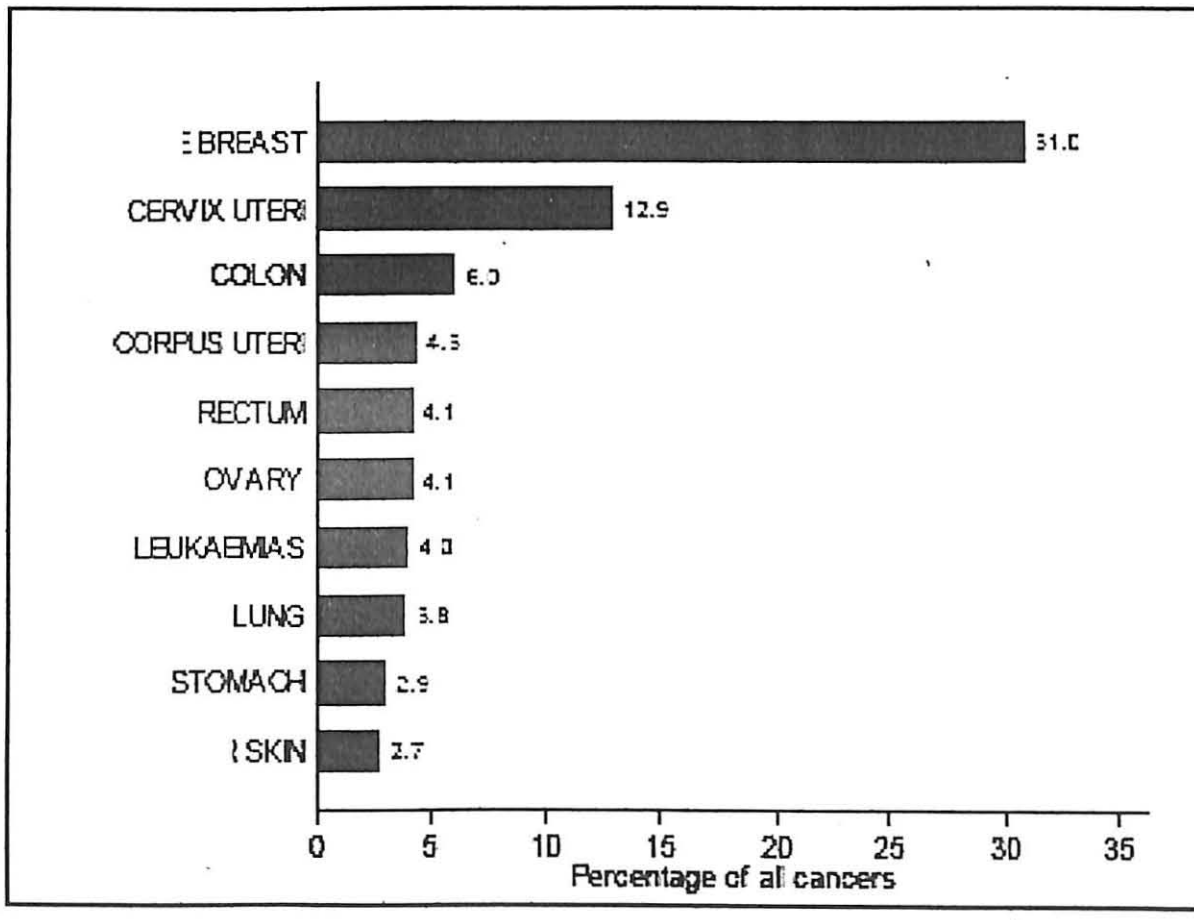

Figure 1.1 Ten most frequent cancers (females) in Peninsular Malaysia for the year 2003 (adapted from Lim *et al.*, 2003).

1.2 Apoptosis

Apoptosis or programmed cell death is the word derived from the ancient Greek, meaning "to fall away from" referring to the falling of leaves in autumn from deciduous trees. The term apoptosis was first introduced by Kerr and his colleagues in 1972 to describe the process of ultrastructural changes characteristic of dying cells. Apoptosis is a process of cell suicide, the mechanisms of which are encoded in the chromosomes of all nucleated cells. It is a crucial process for normal development and maintenance of tissue homeostasis by elimination of damaged, virally infected, or otherwise harmful cells.

Apoptosis is also an efficient method of preventing malignant transformation because it removes cells with genetic lesions. Abnormal apoptosis can thus promote cancer development both by allowing accumulation of dividing cells and by obstructing removal of genetic variants with enhanced malignant potential (Kerr *et al.*, 1972).

Apoptosis is often observed during homeostasis, embryogenesis and during the induction and maintenance of immune tolerance (Sambhara and Miller,1991; Zychlinsky *et al.*, 1991). The characteristics of the apoptotic cell include chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing and cell shrinkage. Eventually, the cells break into small membrane-surrounded fragments (apoptotic bodies), which are then cleared by phagocytosis without inciting an inflammatory response (Reed, 2000).

Apoptosis is an active and tightly regulated process that is induced by the activation of effector proteases called caspases that cleave specific death substrates, resulting in cellular disassembly. Because of the orderly way in which apoptosis takes place, release of inflammatory mediators in the extracellular environment is prevented (Hougardy *et al.,* 2005).

1.2.1 Mechanism of Apoptosis

Apoptosis can take place through two central pathways, one is dependent on mitochondria, known as mitochondrial or intrinsic pathway and one is independent of the mitochondria involving the activation of caspases (cysteinyl-aspartic acid proteases) known as caspase or extrinsic pathway (Figure 1.2) (Sellers and Fisher, 1999).

The mitochondrial pathway is triggered by several stress signals, that includes DNA damage (induced by radiation or chemotherapeutic agents), stress molecules (reactive oxygen species, etc.), or growth-factor withdrawal. These stress signals can trigger the release of pro apoptotic proteins from the mitochondrial intermembrane space into the cytosol. Bcl 2 protein family are the important apoptosis regulators of the intrinsic pathway, in which members are either death antagonists (Bcl 2, Bcl X_L, etc.) or death agonists (Bax, Bak, Bad, Bid, etc.). The ratio of pro apoptotic to anti apoptotic Bcl 2 family proteins establishes the cellular sensitivity to apoptotic signals through the mitochondrial pathway. Activation of the mitochondrial pathway results in the release of cytochrome c and activation of

caspase 9, which then activates caspase 3 and therefore, leading to apoptosis (Hougardy *et al.*, 2005).

The extrinsic or caspase pathway, on the other hand, is initiated by the activation of death receptors (tumor-necrosis-factor-receptor superfamily; TNFR) on the cell membrane. Apoptosis is triggered by the binding of specific tumor-necrosis-factor-receptor superfamily ligands, such as FASL or TRAIL, to their respective receptors, FAS and DR4 or DR5. Death receptor activation results in the formation of an intracellular death-inducing signaling complex (trimerised receptor molecules, FAS-associated death domain molecules and procaspase 8 molecules) which will activate a caspase 8-initiated intracellular apoptotic cascade. This will lead to the cleavage of several substrates in the cytoplasm and nucleus and completion of the apoptotic programme (Hougardy *et al.*, 2005).

Activation of caspase 8 leads to the activation of caspase 3, either directly or indirectly by cleaving Bid to tBid (Figure 1.2) which will trigger mitochondrial apoptotic pathway and ultimately apoptosis.

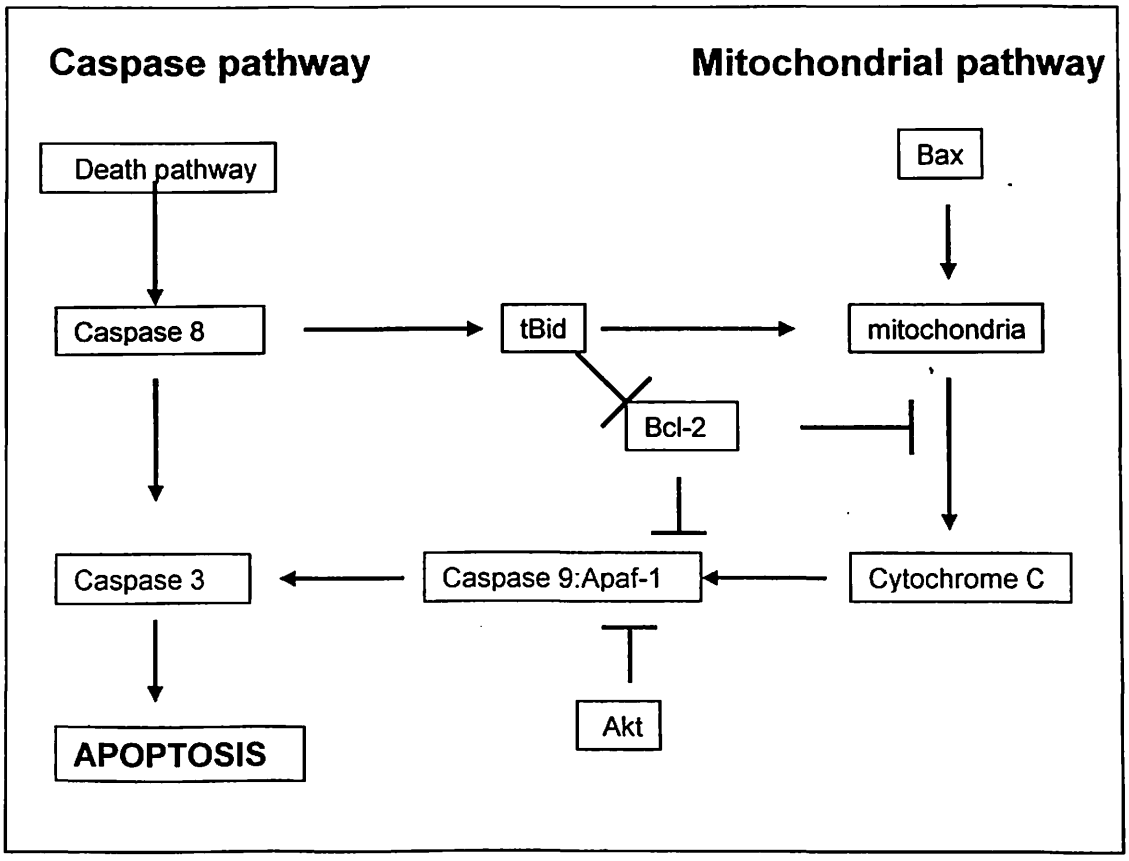

Figure 1.2 The central apoptotic machinery involving the caspase pathway and the mitochondrial pathway (modified from Seller and Fisher, 1999).

The process of apoptosis revolves around the caspases, Bcl 2 protein family and certain anti apoptotic proteins (Akt, etc). Caspase 8 and caspase 9 cleave and activate effector caspase , caspase 3. Certain anti apoptotic proteins can directly or indirectly inhibit caspase 9 activation through Apaf-9 or Akt, which in turn is antagonized by tBid. Thus showing an interconnection between the two pathways.

1.3 Peroxisome proliferator-activated receptors (PPARs)

PPARs were first discovered by Issemann and Green in 1990. They are members of the nuclear hormone receptor superfamily, which function as ligand-dependent, sequence specific activators of transcription (Evans, 1988). PPARs were cloned and firstly characterized as orphan members of the nuclear receptor gene family that includes the receptors for the steroid, retinoid and thyroid hormones (Rosen and Spiegelman, 2001). Subsequently, various ligands (natural and pharmacological) for these receptors were identified (Lehmann *et al.,* 1995).

Encoded by separate genes and characterized by specific tissue and developmental distribution patterns, PPAR are divided into three isoforms namely PPARα, PPARβ or PPARδ and PPARγ (Mangelsdorf *et al.,* 1995). PPARα is expressed in liver, intestine, pancreas, kidney, muscle, heart, skeletal muscle, adrenals and cells from the vascular wall. PPARγ on the other hand, is mainly expressed in adipose tissues where it plays a role in lipid metabolism, in intestine, mammary gland, endothelium, liver, skeletal muscle, prostate, colon and in immune cell types throughout the body, including monocytes and macrophages. PPARδ is expressed in a wide range of tissues, as human embryonic kidney, small intestine, heart, adipose tissue, skeletal muscle and developing brain, with a less-defined function (Theocharis *et al.,* 2004).

PPARγ, the best characterized of the PPARs, participates in a wide range of biological processes such as lipid and glucose metabolism, adipocyte differentiation, inflammatory responses, cell proliferation, apoptosis and cancer

(Lehmann *et al.*, 1995). Therefore, PPARγ has been the central issue in the field of molecular and clinical therapeutics, and it has now become important to determine the role of PPARγ in major human chronic diseases such as atherogenesis and carcinogenesis. Pharmacological components that target PPARγ can be of significant importance for the development of new agents for chemotherapy or chemoprevention of cancer (Sporn *et al.*, 2001).

1.3.1 Structure of PPARγ

PPARγ consists of four functional domains with five structural regions namely A/B, C, D, E and F domains (Figure 1.3).

The amino terminal **A/B domain** is a poorly conserved domain among the three isoforms of PPAR. It is involved in the ligand-dependent transcriptional activation function-1 (AF-1) which is active in some cells. The transcriptional activity of PPAR can be affected by alteration in the phosphorylation of this domain by various signaling pathways (Boitier *et al.*, 2003).

The **C domain** is highly conserved and more frequently known as DNA-binding domain or DBD. The DBD contains two zinc finger patterns which bind to the regulator region of DNA when the receptor is activated. The **D domain** encodes for the flexible hinge region which allows independent movement of ligand-binding domain (LBD) (Boitier *et al.*, 2003).

The **E/F domain** or LBD contains the AF2 ligand-dependent activation domain. LBD has an extensive secondary structure of several alpha helices and a beta sheet. Natural and synthetic ligands bind to the LBD, activating the receptor.

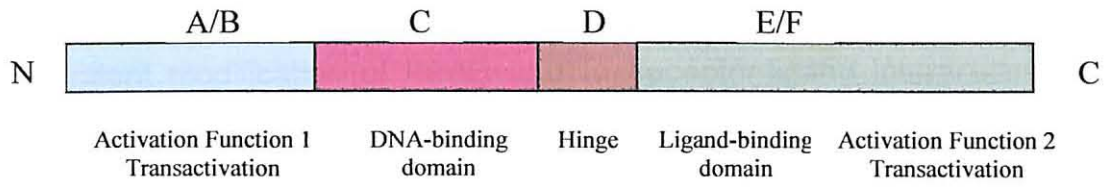

Figure 1.3 A schematic diagram of PPAR domain structure (modified from Boitier *et al.*, 2003).

1.3.2 Ligands for PPARγ

PPARγ is activated by natural (endogenous) or synthetic (pharmalogical) ligands. Endogenous ligands are mostly fatty acids or their derivatives. The most potent endogenous PPARγ activator is 15-deoxy prostaglandin J_2 (15d-PGJ_2), whereas synthetic ligands include the oral antibiotic drugs thiazolidinediones (TZDs), such as ciglitazone, non steroidal anti inflamatory drugs (NSAIDs), etc.

15d-PGJ_2 is a bioactive prostanoid produced by dehydration and isomerization of PGD_2, a cyclooxygenase product. 15d-PGJ_2 is shown to be a potent inducer of caspase-mediated apoptosis in a variety of cells (Bailey and Hla, 1999). There are

reports of the inhibition of tumor-cell growth both *in vitro* and *in vivo* by 15-d-PGJ_2 in a variety of tissues, including breast, prostate, colon, lung, bladder and esophagus.

1.3.3 Transcriptional activation of PPARγ

Activation of PPARγ takes place by three ways (a) heat shock protein 72 pathways, (b) covalent modification of PPAR and (c) receptor-ligand interactions (Vamecq and Latruffe, 1999). PPARγ forms a heterodimer with the retinoid X receptor (RXR). Upon ligand binding, the complex of PPAR and RXR binds to specific recognition sites on DNA, the peroxisome proliferator response elements (PPREs) and regulates transcription of specific genes which will lead to protein synthesis and later, biological effects (Figure 1.4).

1.3.4 The role of PPARγ in cancer

Apoptotic cell death has been reported in a wide variety of (*in vitro* and *in vivo*) cancer models, following treatment with PPARγ ligands (Konopleva and Andreeff, 2002). A variety of natural and synthetic PPARγ ligands sensitize tumor but not normal cells to apoptosis induction by TNF-related apoptosis inducing ligand (TRAIL). PPARγ ligands selectively reduce the levels of FLICE-inhibitory protein (FLIP), an apoptosis-suppressing protein that blocks early events in TRAIL/TNF family death receptor signaling.

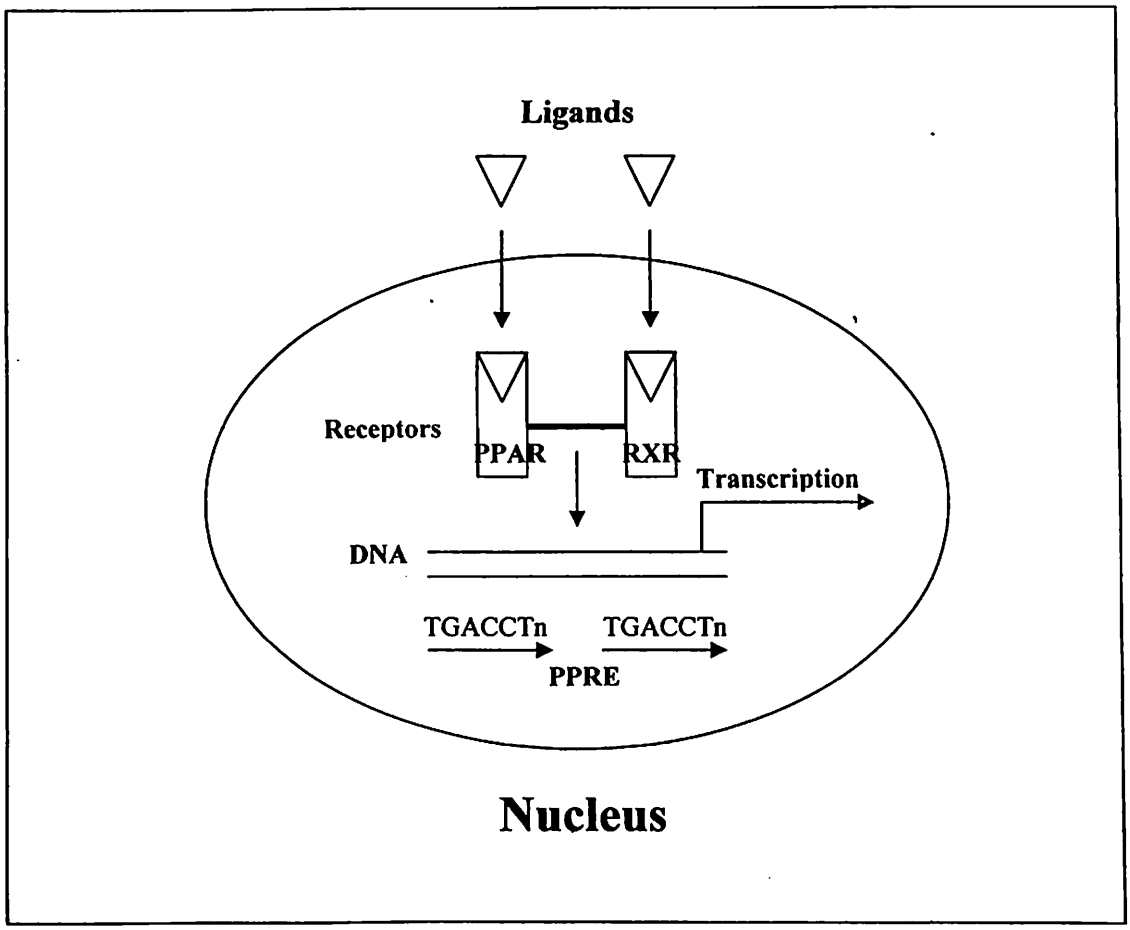

Figure 1.4 Schematic illustration of mechanism of PPAR as ligand-activated transcriptional factor (modified from Theocharis *et al.*, 2004).

Upon ligand binding, the PPAR forms a heterodimeric complex with RXR, that binds to the PPRE and drives the transcription of target genes.