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Bing Ren

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of the requirements for

Ph.D. degree in Biochemistry

Major professor
Donald B. Jump, Ph.D.

Date June 18, 1998

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INVOLVEMENT OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA IN POLYUNSATURATED FATTY ACID REGULATION OF HEPATIC S14 GENE TRANSCRIPTION

By

Bing Ren

A DISSERTATION

Submitted to

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DOCTOR OF PHILOSOPHY

Department of Biochemistry

1998

Donald B. Jump, Ph.D.

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ABSTRACT

INVOLVEMENT OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA IN POLYUNSATURATED FATTY ACID REGULATION OF HEPATIC S14 GENE TRANSCRIPTION

By

Bing Ren

Polyunsaturated fatty acids (PUFA) have dramatic effects on hepatic lipid metabolism by regulating the transcription of specific genes encoding enzymes involved in glycolysis and lipogenesis. The S14 gene, which encodes a putative lipogenic protein, has been used as a model to define the molecular basis of PUFA action on hepatic gene expression. PUFA target *cis*-regulatory elements located between -220 and -80 bp upstream from the 5' end of the S14 gene. Peroxisomal proliferators (PP) also have dramatic effects on hepatic lipid metabolism through effects on gene expression. The mechanism of PP action is mediated through peroxisomal proliferator activated receptor (PPAR). We found that the potent peroxisomal proliferator, i.e. Wy14,643, suppressed $mRNA_{S14}$ and the activity of an S14CAT fusion gene in cultured primary hepatocytes. Wy14,643 and PPARα target the S14 TRR (-2.8 to -2.5 kb). Cotransfection of RXR abrogated the inhibitory effect of PPARα on S14 gene transcription. Further gel mobility shift experiments suggest that Wy14,643 and PPARα interfere with T_3 induction of S14 gene transcription by inhibiting TRβ1/RXR binding to S14 TREs. By using acyl CoA oxidase (AOX) and cytochrome P450 4A2 (CYP4A2) as models for peroxisomal and microsomal enzymes, respectively, and fatty acid synthase (FAS) and the S14 protein,

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models for lipogenic genes, the role of PPARα in PUFA regulation of gene expression was examined. PUFA ingestion induced hepatic AOX and CYP4A2 mRNAs and suppressed FAS and S14 mRNAs in mouse liver. In mice lacking functional PPARα, PUFA did not induce AOX or CYP4A2 mRNAs, indicating a requirement for PPARα in the PUFA-mediated regulation of these genes. In the same PPAR knockout mice, PUFA still suppressed FAS and S14 mRNAs, indicating that PUFA regulation of lipogenic gene transcription does not require PPARα. Additional studies in rats indicate that S14 and AOX are differentially regulated by PUFA. PUFA suppression of $mRNA_{S14}$ preceded the induction of $mRNA_{AOX}$ following PUFA ingestion. Feeding rats with Gemfibrozil induced $mRNA_{AOX}$ 5 to 6-fold, while only marginally affecting $mRNA_{S14}$. Finally, treating primary rat hepatocytes with 18:2, 18:3 (α and γ), 20:4 or 20:5 suppressed $mRNA_{S14}$, but only 20:5 induced $mRNA_{AOX}$. These studies provide evidence for two distinct pathways for PUFA control of hepatic lipid metabolism: one requires PPARα and is involved in regulating peroxisomal and microsomal enzymes; the other mechanism does not require PPARα and is involved in the PUFA-mediated suppression of lipogenic gene expression.

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I feel lucky for having a chance to work in a friendly environment during the past four years. My colleagues, Dr. Annette Thelen, Marya Liimatta, Angela Chapman and Michelle Mater were always my inspiration, enlightenment and living grammar books and dictionaries as well.

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Finally and most importantly, my study and research at MSU was made possible by the constant and unselfish support from my parents and my brother back in China. My dear friend Yi Shi has made my life so joyful. I will never be able to do enough in return for what they did for me.

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5. Fatty A

6. Peroxis

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AF

AOX

aP2

BIEN

bp

CAT

C/EBP

CBP

COUP-T

CTD

CYP450

DBD

DEX

DHEA

DR

EAR-1

EcR

EDTA

EGTA

ER

ETYA

LIST OF ABBREVIATIONS

AF	Transactivation function
AOX	Acyl-CoA oxidase
aP2	Adipocyte fatty acid binding protein P2
BIEN	Bifunctional enzyme
bp	Base pair
CAT	Chloramphenicol acetyl transferase
C/EBP	CCAATT enhancer binding proteins
CBP	C/EBP binding protein
COUP-TF	Chicken ovalbumin upstream promoter transcription factor
CTD	C-terminal repeat domain of RNAP-II
CYP450	Cytochrome P450 supergene family
DBD	DNA-binding domain
DEX	Dexamethasone
DHEA	Dehydroepiandrosterone
DR	Direct repeat
EAR-1	*erb*-A related factor-1
EcR	Ecdysone receptor
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoehyl ether) N, N, N', N'-tetraacetic acid
ER	Estrogen receptor
ETYA	5, 8, 11, 14-eicosatetraynoic acid

FAAR

FABP

FAS

FAT

FATP

FUR

GTF

GR

GST

HDL

HEPES

HETEs

HIV

HNF

HRE

HRR

kb

LBD

LDH

LDL

LPL

LTB_4

FAAR	Fatty acid activated receptor
FABP	Fatty acid binding protein
FAS	Fatty acid synthase
FAT	Fatty acid transporter
FATP	Fatty acid transporter protein
FUR	Far upstream regulatory elements
GTF	General transcriptional factor
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
HDL	High-density lipoprotein
HEPES	4-[2-Hydroxyethyl]-1-piperazine ethanesulfonic acid
HETEs	Hydroxyeicosatetraenoic acids
HIV	Human Immunodeficiency Virus
HNF	Hepatic nuclear factor
HRE	Hormone response element
HRR	Hormone response region
kb	Kilobase
LBD	Ligand-binding domain
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LTB_4	Leukotriene B_4

MAPK

MCAD

ME

MLV

MMTV

MOPs

MR

N-CoR

NF-1

NF-Y

PBS

PEPCK

PK

PPAR

PPRE

PUFA

PUFA-RR

PUFA-RF

RAR

RNAP

RSV

RXR

MAPK	Mitogen-activated protein kinase
MCAD	Medium-chain acyl-CoA dehydogenase
ME	Malic enzyme
MLV	Murine leukemia virus
MMTV	Mouse mammary tumor virus
MOPs	3-[N-Morpholino]propanesulfonic acid
MR	Mineralocorticoid receptor
N-CoR	Nuclear receptor corepressor
NF-1	Nuclear factor-1
NF-Y	Nuclear factor-Y
PBS	Phosphate buffered saline
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pyruvate kinase
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator response element
PUFA	Polyunsaturated fatty acids
PUFA-RR	PUFA response region
PUFA-RF	PUFA regulated factor
RAR	Retinoic acid receptor
RNAP	RNA polymerase
RSV	Rous sarcoma virus
RXR	Retinoid X receptor

S14

SMRT

T_3

TAF

TBP

TK

TR

TRR

USF

VDR

VLDL

S14	Spot 14 protein
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
T_3	3, 5, 3'-triiodothyronine
TAF	TBP-associated factor
TBP	TATA-box binding protein
TK	Thymidine kinase
TR	Thyroid hormone receptor
TRR	Thyroid hormone responsive region
USF	Upstream stimulatory factor
VDR	Vitamin D_3 receptor
VLDL	Very low-density lipoprotein

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INTRODUCTION

"Food is people's real heaven". — Ancient Chinese proverb.

"You are what you eat." — Modern cliché.

Above statements underscore the fact that humans and other animals must obtain their basic organic molecules from food. Fat is an essential macronutrient in the diet of all animals and serves as energy source, components for cell membrane and building blocks of important biomolecules. In both the popular media and scientific press, dietary fat has received considerable attention because of its linkage to a number of chronic diseases and disease-prone physiological status, including insulin-resistance, heart disease, a number of cancers and obesity. Recent studies indicate that fatty acids have pronounced effects on gene expression. These studies may provide important clues to explain how fatty acids contribute to the onset and progression of these chronic diseases.

Our research group focus on the polyunsaturated fatty acid (PUFA) regulation of hepatic fatty acid metabolism. These metabolic processes are subject to complex hormonal and nutritional control. A number of genes involved in these metabolic processes have been cloned, and the molecular basis of transcriptional control delineated. These genes provide excellent model systems to examine how PUFA interact with hormonal regulatory mechanisms either to augment or to abrogate gene transcription.

This dissertation intends to investigate the molecular mechanism by which PUFA inhibit hepatic S14 gene transcription. I will first examine the candidacy of a nuclear receptor, peroxisome proliferator activated receptor α, as the mediator for PUFA action

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on S14 gene, then proceed to the molecular basis of PPAR control of S14 gene transcription, and conclude with a description of mechanisms where fatty acids are known to activate PPAR and regulate hepatic lipogenic and lipolytic genes.

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Chapter 1. Literature Review

Under the guidance of Dr. Jump, I spent the past four years working on the project of defining the role played by peroxisome proliferator activated receptor α (PPARα) in the regulation of hepatic S14 gene transcription by polyunsaturated fatty acids (PUFA). In order to put my research in the relevant biological background, in this chapter, I attempt to provide an overview of general concepts as well as progress that has been made in recent years in this research field.

1. Regulation of eukaryotic gene expression

Gene expression is the realization of the flow of biological information from DNA to RNA to protein. The regulation of gene expression is exerted at each level in the pathway. To our present knowledge, the transcription level is the most important control site of most genes (Wingender, 1993; McKnight, 1996), including S14 gene (Jump, 1989). Therefore in this review I emphasize the regulation of eukaryotic gene expression at the transcriptional level. Just for sake of completeness, I first briefly summarize the controlling events at other levels of gene expression.

At the DNA level, the regulation is achieved by directing the amplification of parts of the genome and changing their availability for the next step in gene expression pathway. Selective gene proliferation and DNA rearrangement leads to pronounced synthesis of particular gene products. For example, protooncogene c-*myc* can be amplified to hundreds of copies by a series of overreplication, nonhomologous exchange and unequal homologous exchange actions (Luscher and Eisenman, 1990). DNA rearrangement is best represented by the recombination of immunoglobulin exons during B-cell maturation

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(Mombaerts et al., 1992; Sinkai et al., 1992). Alteration of the DNA sequence methylation states controls its accessibility for RNA polymerases. Generally speaking, the degree of methylation is inversely correlated with the transcriptional activity of the corresponding gene (Cedar, 1988).

Between the stages of transcription and translation, the regulation is accomplished by controlling the splicing of mRNA precursors and the degradation of mature mRNA (Padgett et al., 1986; Foulkes and Sassone-Corsi, 1992). Regulation of translation then takes place through controlling the availability of translation initiation factors (Wingender, 1993).

Post-translational modifications of protein are means of very fast cellular control through phosphorylation, glycosylation, myristylation/palmitoylation, methylation, acetylation, and ubiquitinylation (Wingender, 1993). The enzymes that carry out these modifications are also subject to regulatory mechanisms, thus constituting complicated networks for the exquisite control of eukaryotic gene expression.

The transcription of protein-encoding genes is catalyzed by RNA polymerase II (RNAP-II) (Lewin, 1994). The DNA sequences that dictate where an RNA polymerase is to land and in which direction it is to transcribe are defined as core promoter elements (Beato and Sanchez-Pacheco, 1996). The initiation of transcription depends on the recognition of the promoters by two types of transcription factors: the basal or general transcription factors (GTFs), and the modulators or sequence-specific transcription factors. GTFs interact with the core promoter elements, and the modulators generally interact with specific sequences located further upstream of the core promoter. GTFs are sufficient to determine RNA polymerase specificity, and to direct low levels of

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transcription, whereas the sequence-specific transactivators act by enhancing or reducing the basal level of transcription (Beato and Sanchez-Pacheco, 1996).

Most of the factors involved in formation of the transcription initiation complex have been identified and cloned. These factors include TFIID, TFIIB, TFIIA, TFIIF, TFIIE, and TFIIH (Buratowski, 1994). TFIID is a multiple protein complex which is composed of a TATA-box binding protein (TBP) and TBP-associated factors (TAFIIs) (Beato and Sanchez-Pacheco, 1996). TBP is a highly conserved protein, particularly at its C-terminal half, which binds to the minor groove of DNA over the region of the TATA box. Binding induces a drastic bend of the DNA (Kim et al., 1993), which seems to be an important component of the site-specific recognition by TBP (Parvin et al., 1995). TBP also specifically interacts with the non-phosphorylated form of C-terminal repeat domain (CTD) of RNAP-II (Usheva et al., 1992), which is the form known to enter the initiation complex (Chesnut, et al., 1992). TFIIB contacts DNA upstream and downstream of the TATA box, as well as TBP, CTD and other transactivators (Lee and Hahn, 1995; Bagby et al., 1995; Usheva et al., 1992; Ha et al., 1993; Lin et al., 1991; Colgan et al., 1993). TFIIF mediates the association of RNAP-II with promoter sequences containing TFIID and TFIIB (Tan et al., 1995). These GTFs, along with RNAP-II, comprise the minimal transcription system, which is able to perform the low level of basal transcription (Beato and Sanchez-Pacheco, 1996).

For cells to respond to addition of sequence-specific transactivators and to perform more efficient transcription, more GTFs are needed (Buratowski, 1994). These additional GTFs include TFIIA, TFIIE, TFIIH and TAFIIs of TFIID. TFIIA helps to stabilize the binding of TFIID to DNA, and allows TFIID to recognize a wider range of

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promoters (Lee et al., 1992). TFIIA also mediates the functions of a number of transactivators, such as viral protein 16 (VP16) (Kobayashi et al., 1995), and transcriptional suppressers (Aso, et al., 1994; Inostroza et al., 1992).

Whereas the TBP-based minimal transcription system is unable to respond to activation by sequence-specific transactivators, systems containing TBP complexed to TAFIIs in TFIID acquire this property and can respond to such factors (Pugh and Tjian, 1990; Dynlacht et al., 1991). A number of TAFIIs have been isolated from human (Timmers and Sharp, 1991; Brou et al., 1993), *Drosophila* (Dynlacht et al., 1991; Kokubo et al, 1993) and yeast (Reese et al., 1994; Poon et al., 1995). It has been shown that *Drosophila* TAFII150 is necessary for transcriptional activation by nuclear factor 1 (NF-1), and that *Drosophila* TAFII110 is required for activation by SP1 and cAMP response element-binding protein (CREB) (Weinzierl et al., 1993; Hoey et al, 1993). Human TAFII55 is known to interact with multiple transactivators, including Sp1, USF and HNF-1 (Chiang and Roeder, 1995).

TAFIIs have also been shown to be essential for a key feature of eukaryotic transcription: the synergism between different transactivators. This is achieved by a multivalent interaction of each of the transactivators with a particular TAF, leading to synergistic recruitment of TFIID to the promoter (Sauer et al, 1995).

In addition to the assembly of the transcription initiation complex, promoter clearance is also a step relevant to the control of transcriptional efficiency. Promoter clearance is a term describing the conversion of an initiation complex into an elongation complex. It is believed that promoter clearance is accompanied by phosphorylation of the CTD of RNAP-II, which leads to its dissociation from TBP (Beato and Sanchez-Pacheco,

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1996). Additional polypeptides are required for this process. They include TFIIE and TFIIH. TFIIE interacts with RNAP-II, TFIID, TFIIF and helps to recruit TFIIH to transcription complex (Lu et al., 1992; Maxon et al., 1994; Ohkuma et al., 1995). TFIIH possesses activities of CTD kinase, ATPase and helicase (Lu et al., 1992; Svejstrup et al., 1995). All these enzymatic activities are required for efficient promoter clearance as well as nucleotide excision repair of DNA (Svejstrup et al., 1995; Akoulitchev et al., 1995).

In vitro transcription data suggest that initiation by RNAP-II in the absence of transcriptional activators is limited by melting of the promoter DNA upstream of the initiation site, as required for the formation of an open complex (Pan and Greenblatt, 1994). After formation of an open complex and synthesis of a few nucleotides, additional controls regulate the rate of effective polymerase elongation. Such elements controlling the elongation step in transcription have been identified in the promoters of c-*myc*, *Drosophila hsp*70 and human immunodeficiency virus-1 (HIV-1) genes (Gilmour and Lis, 1986; Beato and Sanchez-Pacheco, 1996). The c-*fos* promoter is controlled not only by factors binding upstream of the initiation site, but also by intragenic regulatory elements located at the end of the first exon and within the first intron (Lamb et al., 1990; Mechti et al., 1991).

Chromatin structure also participates in transcriptional regulation. Eukaryotic DNA is organized in chromatin within the cell nucleus. The basic unit of chromatin is the nucleosome, which consists of a protein octamer with two each of the histones H3, H5, H2A, and H2B and 145 bp of DNA wound around the octamer (Lewin, 1994). The nucleosomal fiber is further folded into less well defined higher order structures, partly by the cooperative binding of linker histones (Van Holde and Zlatanova, 1995). Structural

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homologies have been found between histones and transcription factors. For instance, winged motif, a structural domain of linker histones, is also present in HNF-3, a transcription factor important for the function of the rat albumin enhancer and for the glucocorticoid induction of the rat tyrosine aminotransferase (TAT) gene (Clark et al., 1993; Brennan, 1993). Histone H2A and H3B show structural homology to the C and A subunits of NF-Y/CBF, a multimeric transcription factor that is involved in CAAT box recognition (Sinha et al., 1995). Histone H3 and H4 exhibit structural homology with TAFII40 and TAFII60, respectively (Thut et al., 1995).

There is also direct evidence supporting a role for chromatin organization in transcriptional control, especially for synergistic interaction between hormone receptors and other transcription factors, as will be reviewed in the following section.

2. Transcriptional regulation by nuclear receptors

Gene transcription responds to environmental stimuli in different ways. Hydrophilic molecules, such as peptide hormones, growth factors and neurotransmitters, bind and activate cell surface receptors, initiating a cascade of intracellular signals by a complex system of secondary messengers. These messengers eventually transmit a signal to *trans*-acting factors that control transcription rates of target genes (Wingender, 1993). Lipophilic signaling molecules, such as certain hormones and vitamins, can enter the cell by simple or facilitated diffusion and transduce the signal to the genome via intracellular receptors. In contrast to the membrane receptors, these intracellular receptors act as transcription factors and exert their regulatory functions directly at the gene level. They belong to the distinct class of nuclear receptors (Evans, 1988).

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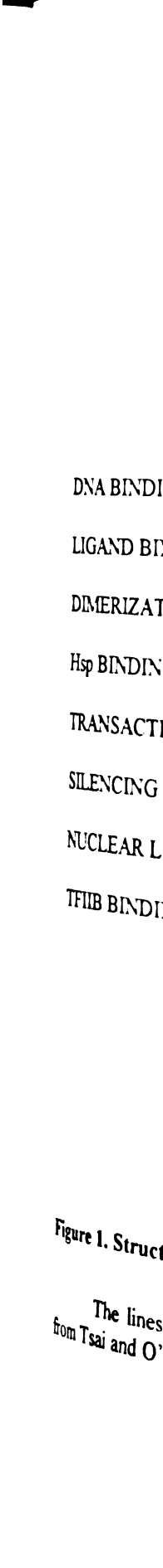
Many nuclear receptors belong to the steroid/thyroid hormone receptor superfamily, which represents the largest known family of transcription factors in eukaryotes (Soontjens et al., 1996). It includes receptors for the steroids, glucocorticoid (GR), progesterone (PR), estrogen (ER), mineralocorticoid (MR), androgen (AR), thyroid hormone (TR), vitamin D_3 (VDR), all-*trans* retinoic acid (RAR), 9-*cis* retinoic acid or retinoic acid X (RXR) and ecdysone (EcR). (Tsai and O'Malley, 1994). In addition, cloning has identified a large number of genes having sequence homology to the steroid/thyroid hormone receptor superfamily, such as chicken ovalbumin upstream promoter transcription factor (COUP-TF). Since the endogenous ligands for the proteins encoded by these genes are not known, they have been collectively termed "orphan receptors" (Evans, 1988). Furthermore, a variety of isoforms of TR, RAR, RXR and PR have been identified. These isoforms are expressed in distinct cell types and developmental stages, suggesting that they play a variety of physiological roles (Soontjens et al., 1996).

Nuclear receptors display a unique domain structure. Figure 1 illustrates the representative structure of a nuclear receptor (Tsai and O'Malley, 1994). Certain domains share close structural homology between different receptors, and others are conserved only between isoform variants of the same receptor. All receptors contain DNA- and hormone-binding domains. The remaining functions are a composite from studies of various receptors, and some receptors may lack specific functional regions. The N-terminal A/B domain is highly variable in sequence and in length. Usually, this domain contains a constitutive transactivation function (AF-1), which is involved in activation of target genes, presumably by interacting with components of the core transcriptional machinery, coactivators, or other transactivators (Godowski et al., 1988; Webster et al.,



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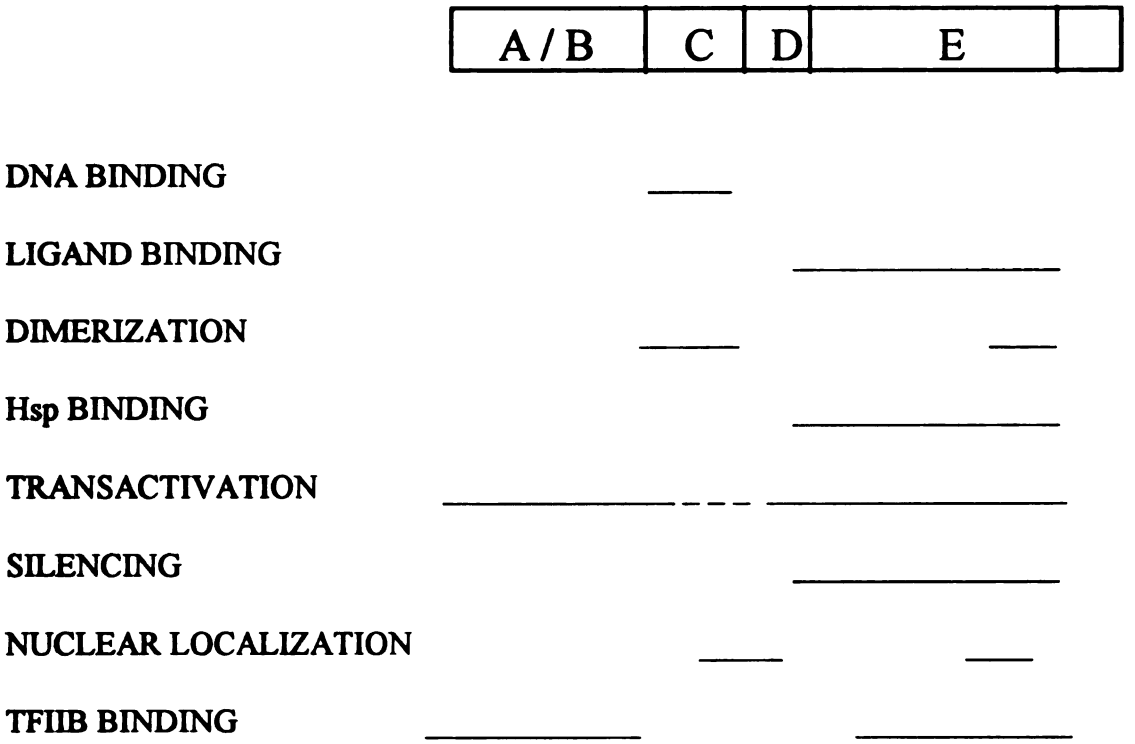

Figure 1. Structure of a typical nuclear receptor

The lines underneath indicate the functions of the corresponding regions (Cited from Tsai and O'Malley, 1994).

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1988; Hollenberg and Evans, 1988). This region is also important for determining target gene specificity for receptor isoforms, which recognize the same response element (Tora et al., 1988; Bocquel et al., 1989). The C region is also known as DNA binding domain (DBD). It contains two type II zinc (Zn) fingers and is the most conserved domain for all nuclear receptors (Schoonjans et al., 1996). (Wingender, 1993).

The D region exists as a hinge region downstream of the C region. This region may allow the protein to bend or alter conformation, and often contains a nuclear localization domain (GR, PR) (Giguere et al., 1986; Picard and Yamamoto, 1987; Evans, 1988; Kumar, 1987) and transactivation domain (TR, GR) (Godowski et al., 1988, Guiochon-Mantel et al., 1989). Located further downstream, the E-region is also named ligand-binding domain (LBD). Unlike other functions which require short stretches of the amino acid sequence, ligand binding appears to involve the majority of the E-region (Soontjens et al., 1996). This region also harbors the functions of heat-shock protein association, dimerization, nuclear localization, ligand-dependent transactivation (AF-2), intermolecular silencing (for TR, RAR, COUP-TF) and intramolecular repression (for PR) (Evans, 1988; Graupner et al., 1989; Fawell et al., 1990; Chambraud et al., 1990; Forman and Samuels, 1990). The variable F region is currently considered dispensable, because no specific function of this region has been identified. For example, deletion of the F-region in ER does not affect any known ER function (Kumar et al., 1987).

DNA sequences responsive to steroid/thyroid hormones have been termed hormone response element (HRE) (Evans, 1988). Almost all HREs are derivatives of a canonical hexameric DNA sequence AGGTCA (Schoonjans et al., 1996). Mutation of the individual nucleotides, duplication of the hexameric sequence, and addition of flanking and

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intervening sequences, have generated distinctive response elements for specific receptors. The nuclear receptors can be classified under four major subgroups according to their DNA binding properties as well as dimerization patterns (Mangelsdorf et al., 1995). Class I receptors consists of the classic steroid receptors which are localized in the cytoplasm. These receptors are associated with heat-shock proteins and upon binding of their cognate ligand translocate to the nucleus. They bind as homodimers to DNA half sites arranged as inverted repeats. Members belonging to this subgroup are GR, AR, PR, MR and ER. Class II receptors consists of nuclear receptors which heterodimerize with RXR and bind to direct repeats (DRs) separated by a variable number of nucleotides. The intervening nucleotides are termed spacers. The localization of this class of receptors is independent of ligand activation. They always exist in nuclei. This class includes TR, RAR, VDR and PPAR, which will be discussed in more detail in section 7. More importantly, class II receptors bind to their cognate HREs with higher affinity as heterodimers with RXR than as homodimers (Tsai and O'Malley, 1994). Class III receptors bind to direct DNA repeats as homodimers and include RXR, HNF-4 and COUP-TF. Class IV receptors, bind as monomers to a single hexameric core recognition motif flanked by some additional sequences upstream of this motif. This last class of receptors include Rev-erbα (EAR-1), Rev-erbβ (RVR), NGF1-B and FTZ-F1α, β.

In vitro binding and *in vivo* transfection studies have defined the preferred HREs for many nuclear receptors. However, receptors show a considerable freedom to bind and activate different response elements, albeit with variable affinity (Hwung et al., 1988; Cooney et al., 1992). For example, TR preferentially bind to DR-4, but is also found to bind DR-2 with a lower affinity. COUP-TF binds with reasonable affinity to the AGGTCA

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direct repeat with a spacer of anywhere from 1 to 10 nucleotides. It also binds to inverted AGGTCA repeats with different spacers. It has been proposed that this promiscuous binding allows a more subtle regulation of the target genes, and eventually contribute to the specific phenotype of the cell (Green, 1993).

In transfections and in cell-free transcription assays, nuclear receptors can activate transcription from minimal promoters in the presence of their cognate ligands (Schat et al., 1990; Elliston et al., 1990). The activation appears to be mediated by the interaction of nuclear receptors with several components of the general transcriptional machinery. TBP, TFIIB and TAFIIs have been identified as potential targets of hormone receptors. ER transactivation is enhanced in response to overexpression of TBP and the two proteins interact *in vitro* (Sadovsky, et al., 1995). hTAFII30 is required for ER transactivation and interact with ER *in vitro* (Jacq et al., 1994). dTAFII110 has been shown to interact with PR (Schwerk et al., 1995), RXR and TR (Schulman et al., 1995). A hormone-independent interaction with TFIIB has been well documented for TR (Baniahmad et al., 1993, Tone et al., 1994) and for VDR (MacDonald et al., 1995; Blanco et al., 1995).

The interaction between the nuclear receptors and the basal transcription machinery can be either direct or indirect, e.g. via transcription-intermediary factors (TIFs). TIFs are also termed adaptors, coactivators or cointegrators by different researchers. There is a long list of TIFs that have been shown to interact with different nuclear receptors in *in vitro* binding assays (Beato and Sanchez-Pacheco, 1996). For example, SUG1, a component polypeptide of the RNAP-II holoenzyme, has been shown to interact with TR (Lee et al., 1995) and RAR (vom Baur et al., 1995). Members of the CBP/P300 family bind to nuclear receptors ER, RAR, RXR, VDR and TR in a ligand-

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dependent manner. Overexpression of CBP/P300 is sufficient to augment transactivation by these receptors in transfection studies (Kamei et al., 1996; Hanstein et al., 1996). CBP/P300 family is known to interact with components of the basal transcription apparatus (Abraham et al., 1993; Kee et al., 1996). It is proposed that by these direct and indirect interactions with the transcription machinery, nuclear factors help to increase the rate of preinitiation complex formation, or stabilize the formation of a preformed preinitiation complex, and thus facilitate gene transcription (Tsai and O'Malley, 1994; Kamei et al., 1996; Hanstein et al., 1996).

In the absence of hormones, TR and RAR bind to their HREs and actively repress promoter activity through a mechanism termed silencing (Levine and Manley, 1989; Renkawitz, 1990). Studies with truncation mutants of the receptors have indicated that the silencing activity is located within the LBDs of these receptors (Tong et al., 1995). The silencing activity of TR can be reversed by coexpression of the LBD of TR, suggesting the existence of a cellular corepressor which is limiting within the cell (Baniahmad et al., 1995). Furthermore, coexpression of the LBD of RAR or glutathione S-transferase (GST) also greatly reduce the TR-mediated silencing, indicating that the RAR and GST LBDs may bind to the same corepressor as the TR LBD (Tong et al., 1996). By using the yeast two-hybrid screening system, a novel family of nuclear receptor corepressors have been identified. These receptor-interacting proteins include the nuclear receptor corepressor (N-CoR) (Horlein et al., 1995) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT) (Chen et al., 1995). These factors bind to the hinge regions of TR and RAR, and repress the transactivation by TR and RAR through an unknown mechanism. When the ligands for TR and RAR are present, the corepressor is released, relieving

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repression and permitting transcriptional activation. AF2 in the E regions of TR and RAR appear to be necessary for releasing corepressor (Baniahmad et al., 1995; Chen and Evans, 1995), as well as activating transcription (Danielian et al., 1992; Barettino et al., 1994; Durand et al., 1994).

Structural and biochemical studies suggest that, besides displacement of negative corepressors, such as in the cases with TR and RAR, hormone binding induces a conformational change in the receptor, which results in either exposure of the dimerization domain for binding other coactivators (Fritsch et al., 1992; Allan et al., 1992; Beekman et al., 1993; Leng et al., 1993; Toney et al., 1993), or inactivation of the suppressor function within a receptor (Tsai and O'Malley, 1994).

For some nuclear receptors, ligand-dependent activation is enhanced by receptor phosphorylation. PR (Denner et al., 1990; Poletti and Weigel, 1993), ER (Denton et al., 1992; Ali et al., 1993; Le Goff et al., 1994) and GR (Orti et al., 1992) all exhibit enhanced phosphorylation in response to hormone treatment. Functional correlation studies have shown hormone-induced phosphorylation of these receptors precedes gene activation, both *in vivo* (Beck et al., 1992; Weigel et al., 1993) and *in vitro* (Bagchi et al., 1992). These receptors usually exhibit a reduced transactivation activity when the phosphorylation sites are replaced by site-directed mutagenesis (Bai and Weigel, 1996; Jewell et al., 1995; Le Goff et al., 1994; Lahooti, et al., 1995). Other receptors have also been studied with respect to their ligand-dependent phosphorylation, albeit not as extensively as the above mentioned ones. For example, TR (Lin et al., 1992; Sugawara et al., 1994), RARα and RXRα (Lefebvre et al., 1995) all show stronger DNA binding activity in the phosphorylated forms.

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In addition to the putative synergistic effect of phosphorylation on ligand-dependent activation of receptor, several reports indicate that agents that stimulate intracellular phosphorylation pathways can also activate receptors in the absence of their cognate ligands. PR can be transcriptionally activated by 8-bromo-cAMP, okadaic acid (Denner et al., 1990), dopamine (Power et al., 1991) or epidermal growth factor (Zhang et al., 1994). Both ER and AR can be activated by EGF (Aronica et al., 1993; Culig et al., 1994). These findings suggest the existence of cross-talk between signal transduction cascades and steroid/thyroid hormone receptors in producing a biological response. CBP/P300 appears to play an important role in the cross-talk because of its involvement in transactivation by nuclear receptors, such as GR, RAR, RXR, VDR and TR, and in pathways regulated by CREB, AP-1 and mitogen-activated protein kinase (MAPK) (Kwok et al., 1994; Arias et al., 1994; Kamei et al., 1996; Hanstein et al., 1996).

Many eukaryotic genes are under the control of multiple hormones and environmental stimuli. Consequently, HREs are usually found in multiple copies or clustered with other *cis*-acting elements. HREs often interact synergistically with other HREs or with unrelated *cis*-acting elements. Synergism is believed to be mediated by cooperative binding of the nuclear receptors and other *trans*-acting factors that recognize these *cis*-acting elements. Binding of one receptor complex may facilitate the binding of a second receptor. This synergistic interaction allows both complexes to bind with greater affinity and consequently in greater occupancy of the cis-acting elements, thus promoting greater transcription activity (Ptashne, 1988).

Chromatin organization could determine the general accessibility of regulatory sequences on DNA. Genetic and biochemical analyses have provided insight into the role

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of the primary level of chromatin organization in the nuclear receptor control of gene transcription. The mouse mammary tumor virus (MMTV) promoter is transcriptionally controlled by steroid hormones, in particular glucocorticoids and progestins (Truss and Beato, 1993). The hormone receptors bind to a hormone-responsive region (HRR) and facilitate the interaction of other transcription factors, including NF-1, CTF1 and OTF1/OCT-1 (Beato, 1991). *In vitro* nucleosome reconstitution studies have revealed that when the MMTV promoter is packed into chromatin structure, the binding of NF-1 is precluded (Chavez et al., 1995). Nucleosome depletion leads to higher accessibility of the promoter for NF-1 binding (Candau et al., 1996). Hormone induction is believed to cause a displacement or disruption of the nucleosome over the HRR, shown by hypersensitivity of the region to DNase I (Zaret and Yamamoto, 1984), which would facilitate access of NF-1 to its binding site and transcriptional activation (Cordingley et al., 1987). Similar nucleosome disruption mechanisms have been described for the hormonal induction of the tyrosine amino transferase gene (Becker et al., 1984) and of the S14 gene (Jump, 1989a) in rat liver.

3. Nutritional and hormonal regulation of lipogenesis

Carbohydrate, amino acids and fatty acids have been implicated as mediators of transcription regulation (Berdanier and Hargrove, 1993). Because my studies deal with fatty acid regulation of gene transcription, I will focus this discussion on fatty acid effects.

Fatty acids are essential for the function of all cells because they serve as components of biological membranes, precursors of hormones and other intracellular

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messengers, and fuel molecules. Fatty acids are stored as triglycerides. The conversion of glucose to triglyceride is defined as lipogenesis (Hillgartner et al., 1995).

Fatty acids are synthesized in the cell cytosol. For rodents and human, liver is a major organ of fatty acid synthesis. Figure 2 illustrates the pathways involved in conversion of monosaccharides to fatty acids (Hillgartner et al., 1995). Glycolysis and citric acid cycle give rise to acetyl-CoA, the principal building block of *de novo* lipogenesis, and glycerol. NADPH is the hydrogen donor for the following reduction reactions and is generated by the actions of malic enzyme, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Acetyl-CoA is first activated to malonyl-CoA by acetyl-CoA carboxylase. This reaction is the committed step in fatty acid synthesis. The overall synthesis of fatty acids is catalyzed by the fatty acid synthase (FAS) complex, a single polypeptide containing seven distinct enzymatic activities. The condensation and reduction reactions are successively repeated until formation of a palmitate molecule is achieved (Hellerstein et al., 1996).

The amount and composition of macronutrients in the diet, particularly fat and carbohydrate, regulate the rate of fatty acid synthesis. Starvation and high carbohydrate refeeding after long-term starvation result in the lowest and highest rates of lipogenesis, respectively. Animals fed diets that are high in carbohydrates and contain little or no fat have higher rates of lipogenesis than those fed diets rich in fat and low in carbohydrates (Girard et al., 1994). The suckling-weaning transition is also accompanied by a profound change in lipogenesis (Girard et al., 1992).

Changes in nutrient intake are communicated to the cells by circulating hormones and other humoral factors. Altered concentration of these mediators signal the liver and

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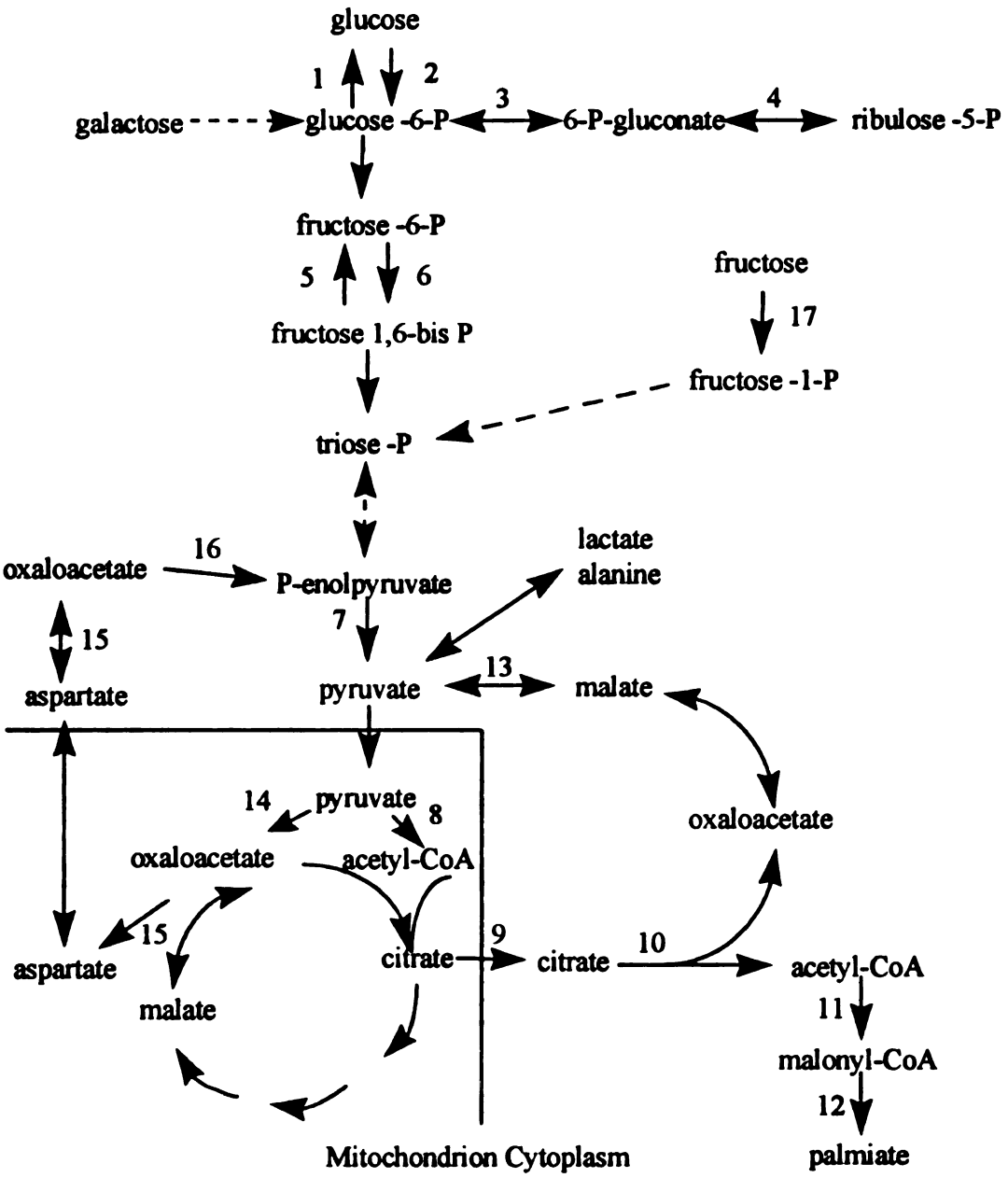

Figure 2. Schematic representation of pathways in conversion of monosccharides to fatty acids in liver

Key reactions are identified by numbers. 1, Glucokinase; 2, glucose-6-phosphatase; 3, glucose-6-phosphate dehydrogenase; 4, 6-phosphogluconate dehydrogenase 5, 6-phosphofructo-1-kinase; 6, fructose-1,6-bisphosphatase; 7, pyruvate kinase; 8, pyruvate dehydrogenase; 9, mitochondrial tricarboxylate anion carrier; 10, ATP citrate lyase; 11, acetyl-CoA carboxylase; 12, fatty acid synthase; 13, malic enzyme; 14, pyruvate carboxylase; 15, aspartate aminotransferase; 16, phosphoenolpyruvate carboxykinase; 17, fructokinase (Cited from Hillgartner et al., 1995).

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other organs to increase or decrease the activities of metabolic enzymes and proteins that regulate the transport, storage, and catabolism of endogenous and exogenous nutrients. With respect to lipogenesis, insulin (Ashcoroft, 1976; Topping and Mayes, 1982) and thyroid hormone (Merimee and Fineberg, 1976; Kaplan and Utiger, 1978; Mariash et al., 1980) are positive effectors; elevated levels of these hormones are characteristic of the fed state. Glucagon is a negative effector; elevated levels are characteristic of the starved state (Goodridge and Adelman, 1976; Witters et al, 1979). These hormones are likely the initial signals because their responses to the onset of nutrient status changes are large and rapid. Furthermore, the circulating levels of these hormones correlate positively (for insulin and thyroid hormone) or inversely (for glucagon) with rates of lipogenesis and the activities of the lipogenic enzymes in liver.

Glucocorticoids (Berdanier and Shubeck, 1979), growth hormone (Schaffer, 1985), and other growth factors (Yoshimoto et al., 1983; Stapleton et al., 1990) also regulate the activities of lipogenic enzymes under some conditions, but they are not considered the major regulators because their responses to dietary changes are not as rapid and significant as those of insulin and glucagon (Hillgartner et al., 1995).

The hormones interact with each other, coordinately and synergistically regulating lipogenic enzyme activities. For example, the inhibition of glucagon secretion by glucose is amplified by insulin (Hillgartner et al., 1995); both insulin and glucagon are involved in the regulation of conversion of thyroxine to the active form 3, 5, 3'-triiodothyronine (T_3) (Mitchell and Raza, 1986).

Hormones are essential extracellular signaling molecules to sense the nutrient intake, but other factors derived from the diet or from mobilization of stored fuels also

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play important regulatory roles. These factors include glucose, fructose and fatty acids. Dietary carbohydrate is a logical candidate for involvement in the signaling of nutritional status because the concentration of blood glucose increases rapidly and dramatically following a high-carbohydrate meal, and during the transition from the starved to the fed state (Randle et al., 1968; Niewoehner et al., 1977). Dietary fructose restored the activities of lipogenic enzymes in streptozotocin-induced diabetic rats (Baker et al., 1952; Volpe and Vagelos, 1974; Fukuda et al., 1992). Rats fed fructose show higher rates of hepatic fatty acid synthesis than the control group fed glucose (Volpe and Vagelos, 1974). In cultured hepatocytes, incubation with glucose and insulin stimulate pyruvate kinase (Decaux et al., 1989) and acetyl-CoA carboxylase gene expression (Katz and Ick, 1981; Salati and Clarke, 1986). Fructose and galactose also potentiate the insulin induction on malic enzyme activity (Mariash and Oppenheimer, 1983). Under physiological concentrations, fructose does not stimulate insulin secretion (Randle et al., 1968). Therefore it is proposed that carbohydrate is the primary regulatory signal for the lipogenic enzymes and that other hormones, like T_3 and glucagon, attenuate this signal (Mariash and Oppenheimer, 1983).

Fat is another macronutrient that regulates lipogenesis. Dietary fats, particularly those rich in long-chain ($C \geq 18$) polyunsaturated fatty acids (PUFA), inhibit the activity of lipogenic enzymes and reduce the rate of fatty acid synthesis (Allmann and Gibson, 1969; Aarsland et al., 1990; Clark et al., 1990). These PUFAs are required to have at least two double bonds located at the 9 and 12 positions to specifically inhibit lipogenic gene expression (Clarke and Clarke; 1982; Clarke and Jump, 1993). In contrast to PUFA,

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The key enzymes involved in the conversion of glucose to fatty acids are regulated by both short- and long-term mechanisms. Short-term changes are initiated by altering the catalytic efficiency of enzymes. Long-term adjustments of enzyme activity generally involve changes in the concentrations of regulatory enzymes.

Short-term control is achieved by covalent modifications, such as phosphorylation-dephosphorylation, and allosteric regulation. In general, starvation leads to increased phosphorylation of lipogenic enzymes, such as acetyl-CoA carboxylase (Thampy and Wakil, 1988; Moir and Zammit, 1990), pyruvate dehydrogenase (Denyer et al., 1986; Holness and Sugden, 1990) and L-type pyruvate kinase (Kohl and Cottam, 1976; Claus, et al., 1984), and causes inhibition of their activities. Conversely, high-carbohydrate diet results in dephosphorylation of these enzymes, which is associated with increased enzymatic activities. Allosteric regulators arise from metabolic intermediates in the lipogenic pathway and function as feed-forward activators or feedback inhibitors. For example, pyruvate inhibits the catalytic efficiency of pyruvate dehydrogenase kinase (Reed and Yeaman, 1987). Pyruvate kinase is allosterically regulated positively by 6-phosphogluconate (Smith and Reedland, 1979; Smith and Reedland, 1981) and negatively by alanine and ATP/ADP ratio (El-Maghrabi et al., 1982).

Long-term regulation of lipogenesis, i.e. alteration of lipogenic enzyme concentration, occurs when the dietary or hormonal stimuli are sustained for prolonged periods. The concentrations of most lipogenic enzymes are regulated by controlling the rate of protein synthesis (Hillgartner et al., 1995). The rate of protein synthesis is

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regulated by controlling the concentration of the mRNA encoding the protein (pretranslational control) or the mRNA translation efficiency (translational control). With respect to lipogenic enzymes, in most cases regulation of protein synthesis is pretranslational by controlling the mRNA abundance (Hillgartner et al., 1995). In this review, I focus on the nutrient and hormonal regulation of two representative lipogenic genes: L-type pyruvate kinase (PK) and fatty acid synthase (FAS).

Increase in level of PK mRNA is induced by insulin and glucose (Decaux et al., 1989) and inhibited by hormones elevating hepatic cAMP levels (Munnich et al., 1984) and polyunsaturated fatty acids (PUFA) (Liimatta et al., 1994). These regulations are mediated primarily by changes in the rate of transcription initiation (Noguchi et al., 1985; Vaulont et al., 1986; Bergot et al., 1992; Liimatta et al., 1994), although posttranscriptional control is also involved in some cases (Vaulont et al., 1986).

The rat L-pyruvate kinase gene contains two promoters: a distal promoter is specific for erythroid cells; and a proximal promoter (L) is specific for liver (Noguchi et al., 1987). Deletion analysis and linker-scanning mutagenesis have localized the regulatory elements responsible for insulin, glucose and polyunsaturated fatty acids between -183 and +11 bp relative to the transcription start site of the L-PK promoter (Cuif et al., 1992; Noguchi et al., 1993; Liimatta et al., 1994). These elements are known to bind liver-specific factors, such as hepatocyte nuclear factor-4 (HNF-4), and ubiquitous transcription factors, such as c-*myc*/USF related factors and nuclear factor 1 (NF-1) (Vaulont et al., 1989). Both c-*myc*/USF related factors- and HNF-4 binding elements are required for the positive control by insulin and glucose, and negative control by glucagon, of the PK promoter (Bergot et al., 1992; Liu et al., 1993). The c-*myc*/USF related factors-binding

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region can also function by itself in multiple copies. HNF-4 binding region alone or in multiple copies is not sufficient to confer either control and is considered to play an ancillary role in the hormonal and nutrient regulation of L-PK gene transcription (Liu et al., 1993). The PUFA responsive elements have been localized in the vicinity of the HNF-4 binding site, suggesting that PUFA-mediated inhibition of L-PK gene transcription occurs via a distinct regulatory pathway and may not represent an interference with glucose and insulin signaling (Liimatta et al, 1994).

FAS activity is not known to be regulated by allosteric effectors or covalent modification. Dietary and hormonal regulation of FAS activity is always paralleled by the amount of fatty acid synthase in the cell (Moustaid et al., 1993). Changes in fatty acid synthase concentration is mediated primarily by controlling transcriptional activity of the gene (Hillgartner et al., 1995). In both the intact animals and in cultured hepatocytes, the FAS mRNA levels are induced by carbohydrate (Clarke et al., 1990), insulin (Kurtz and Wells, 1981), and T_3 (Mariash et al, 1980; Giffhorn-Katz and Katz, 1986), and inhibited by glucagon and cAMP (Paulauskis and Sul, 1989). Insulin and T_3 exert their induction effects on the transcription of FAS gene in a synergistic manner (Fischer and Goodridge, 1978). The ability of T_3 to stimulate transcription of the fatty acid synthase gene in chick embryo hepatocytes requires the presence of glucocorticoid (Roncero and Goodridge, 1992) and is blocked by protein synthesis inhibitors, such as puromycin and pactamycin, and by protein phosphorylation inhibitors (Wilson et al, 1986; Swierczynski et al., 1991). Thus the T_3 induced FAS transcription requires on-going protein synthesis and on-going protein phosphorylation and may require the presence of a glucocorticoid-sensitive factor.

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The rat and goose FAS genes along with their 5'-flanking regions have been isolated and partially characterized (Amy et al., 1990; Kameda and Goodridge, 1991). An insulin responsive region has been localized within the first 332 bp from the transcription start site (Moustaid et al., 1993), and a carbohydrate response element (ChoRE) has been localized in the first intron of the rat FAS gene (Foufelle et al., 1995).

In summary, insulin, glucagon, T_3, glucose and fatty acids are potential humoral signals that may communicate nutritional status to lipogenic organs. The blood levels of insulin, T_3, and glucose are increased in the fed state and decreased in the starved state; glucagon and fatty acids are increased in the starved state and decreased in the fed state. The changes in the levels of these humoral factors cause both short- and long-term changes in the activities of lipogenic enzymes. Elucidating the mechanisms by which the nutritional and hormonal factors are transduced to and within the cells is an important area of metabolic research.

4. The S14 gene model

The S14 gene encodes a 17 kD protein of 150 amino acids, originally discovered in rat liver (Seelig et al., 1981, 1982). The rat S14 gene is present at 1 copy per haploid genome. The gene is 4.4 kb long and contains 2 exons and 1 intron (Narayan et al., 1984; Liaw and Towle, 1984). Two mRNA species of 1.2 and 1.37 kb are observed in rat due to the presence of 2 polyadenylation signals in the 3' exon of the S14 gene (Liaw and Towle, 1984). The half-life of the S14 mRNA is approximately 90 minutes in rat liver (Kinlaw et al., 1987) and mouse 3T3-F442 cells (Lepar and Jump, 1989), and 5 hours in cultured hepatocytes (Mariash et al., 1984).

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Changes in developmental and nutritional status affect the expression of S14 gene. Transcription of the S14 gene is induced by weaning and high-carbohydrate feeding, and suppressed by starvation and high-fat diet (Jump et al., 1988, 1990, 1993; Hamlin et al., 1989). Hormones T_3 (Narayan et al., 1984; Jump, 1989a), insulin (Jump et al., 1990a), glucocorticoids (Lepar and Jump, 1989; Jump et al., 1992), retinoic acid (Lepar and Jump, 1992; MacDougald and Jump, 1992) induce and glucagon inhibits S14 gene transcription (Jump et al., 1990a). The expression of S14 gene is also regulated by tissue-specific factors. S14 mRNA (Jump, 1989) and protein (Kinlaw et al., 1989) are expressed at high levels in tissues involved in lipid metabolism, such as liver, adipose tissue and lactating mammary gland. This tissue-specific regulation of S14 gene expression is at the transcriptional level (Jump, 1989).

The precise function of S14 protein remains unknown. A body of circumstantial data supports the view that S14 protein may play a role in lipogenesis. These data include 1) S14 mRNA and protein are abundantly expressed only in lipogenic tissues(Jump, 1989; Kinlaw et al., 1989); 2) hepatic S14 is expressed at very low level until the rat is weaned on to a chow diet (Jump and Oppenheimer, 1985); 3) the hepatic response of S14 gene to thyroid hormone, insulin, glucocorticoids, adrenergic agonists and glucagon is very similar to the responses of genes encoding lipogenic enzymes, such as FAS, L-PK and malic enzyme; 4) S14 gene expression responds to starvation-refeeding transition, high-carbohydrate and high-fat diet manipulation in the same way as lipogenic genes (Clarke et al., 1990); and 5) Hepatic content of the S14 mRNA exhibits a circadian variation that matches that of lipid synthesis (Jump et al., 1984).

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Immunohistochemical experiments have revealed that S14 is primarily localized in the nuclei of lipogenic tissues (Kinlaw et al., 1992). The induction of S14 mRNA by T_3 and high-carbohydrate diet show the same zonation pattern as that of acetyl-CoA carboxylase (Kinlaw et al., 1993). Inhibition of the expression of the S14 gene by antisense oligonucleotide transfection blocks the induction of ATP-citrate lyase, malic enzyme, L-pyruvate kinase and fatty acid synthase mRNA by T_3 and glucose in cultured hepatocytes (Kinlaw et al., 1995). Transfection experiments indicate that these effects are mediated at the transcriptional level (Brown et al., 1997). These data directly link the function of the S14 protein in the regulation of hepatic lipogenesis and suggest that the S14 protein may play a role in the transduction of hormonal and dietary signals for increased lipid metabolism.

The structure of S14 gene has been extensively studied. Figure 3 shows the major *cis*-regulatory elements located in the 5' flanking region of the S14 gene. The proximal promoter region of the first 300 bp contains elements involved in both constitutive and tissue-specific initiation of S14 gene transcription (MacDougald and Jump, 1991). These elements bind ubiquitous transcription factors nuclear factor-1 (NF-1) and nuclear factor-Y (NF-Y), as well as tissue specific factor C (TSF-C). A PUFA responsive region has been localized within the -80 to -220 bp of the S14 proximal promoter (Jump et al., 1993).

Carbohydrate response region (CHO-RR) is located between -1.6 and -1.4 kb from the transcription start site. In liver, the region also harbors targets for insulin and carbohydrate induction of S14 gene transcription. A carbohydrate response element (ChoRE) has been localized between -1448 and -1428 bp (Shih and Towle, 1995). The element contains a (5')CACGTG motif, which is also present in the ChoRE of L-PK gene

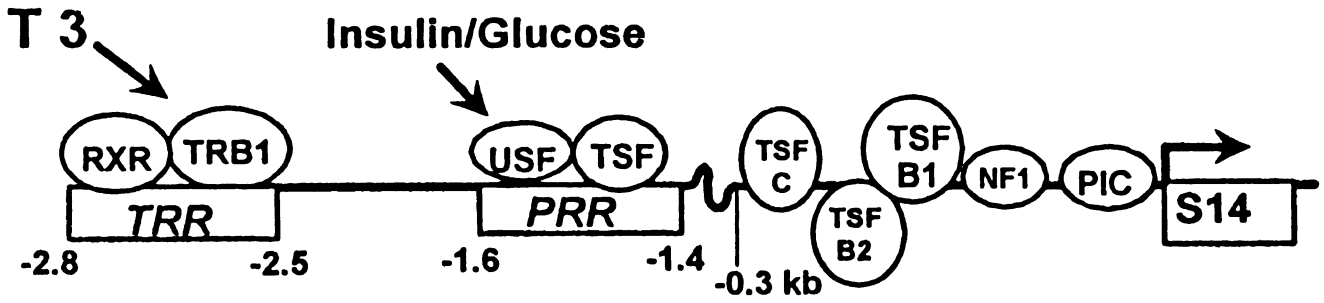

Figure 3. Functional elements controlling hepatic S14 gene transcription

RXR: Retinoid X receptor; TRβ1: Thyroid hormone receptor β1; TRR: Thyroid hormone response region; USF: Upstream stimulatory factor; TSF: Tissue-specific factor; CHO-RE: Carbohydrate response elements; C: Tissue-specific factor C; NF-Y: Nuclear facrtor Y; NF1: Nuclear factor 1; PIC: Pre-initiation complex.

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promoter (Bergot et al., 1992; Liu et al., 1993). This motif is recognized by the c-*myc* transcription factor family, including upstream stimulatory factor (USF) and adenovirus major late transcription factor (MLTF). However, replacement of the motif with authentic MLTF binding site from the adenovirus major late promoter failed to elicit the glucose response on S14 transcription, indicating the requirement for additional DNA sequence flanking the (5')CACGTG motif (Shih and Towle, 1995). In adipocytes, this region also harbors targets for glucocorticoid and retinoic acid regulation on S14 transcription. This region also binds tissue specific factors (MacDougald et al., 1992).

Thyroid hormone response region (TRR) is located between -2.8 and -2.5 kb from the transcription start site. This region contains three thyroid hormone response elements (TRE) that bind thyroid hormone receptor (TR) homodimer and heterodimer of TR and retinoid X receptor (RXR) (Zilz et al., 1990; Liu and Towle, 1994). Each TRE is related to the consensus monomer binding motif 5'-AGGTCA. In two of the elements, these motifs are arranged as direct repeats with 4 base pair spacing (DR-4), while the other one shows a more complex arrangement. Mutagenesis experiments have demonstrated that all three elements are involved in the responsiveness of the S14 gene transcription to T_3 and they act synergistically. At least two elements are required for T_3 induction in hepatocytes (Liu and Towle, 1994).

Because of the extensive knowledge people have gained from S14, with respect to its hormonal, nutritional and developmental control at the molecular level, and because of the unavailability of the same level of information about other lipogenic genes, S14 gene has been used as a model system to investigate the regulation of lipogenic gene expression and proven to be useful. For example, the studies on the S14 gene have contributed to the

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discoveries of the carbohydrate response elements of FAS (Foufelle et al., 1995) and ATP-citrate lyase gene (Kim et al., 1996).

5. Fatty acid β-oxidation and peroxisomal lipid metabolism

The initial event in the utilization of fat as an energy source is the hydrolysis of triglyceride by lipases in adipose tissue, generating free fatty acids and glycerol (Stryer, 1988). Fatty acids are degraded in mitochondria and peroxisomes via a similar mechanism of β-oxidation, consisting of four consecutive reactions: (1) an initial oxidation step in which a 2-*trans*-enoyl-CoA intermediate is formed; (2) a hydration step in which the 2-*trans*-enoyl-CoA is converted to L-3-hydroxyacyl-CoA; (3) a second oxidation step in which L-3-hydroxyacyl-CoA is dehydrogenated to 3-ketoacyl-CoA; and (4) a last reaction in which 3-ketoacyl-CoA is cleaved to acetyl-CoA, which is released, and an acyl-CoA two carbon atoms shorter than the original molecule which then re-enters the β-oxidation spiral (Mannaerts and Van Veldhoven, 1993). In this review, I will focus on the peroxisomal β-oxidation and other roles peroxisomes play in lipid metabolism.

Peroxisomes are single membrane-bound organelles that are present in all eukaryotic cells except for mature erythrocytes (Mannaerts and Van Veldhoven, 1993). When first discovered in the late 50's, the organelles were described by the name "microbody" (Rhodin, 1954; Rouiller and Bernhard, 1956). The name "peroxisome" was coined to emphasize the organelle's role in hydrogen peroxide metabolism (de Duve and Baudhuin, 1966). Glyoxysomes of some plants and yeasts, and glycosomes of trypansomatids are also members of the peroxisome family (Reddy and Mannaerts, 1994).

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To date, more than 50 enzymes have been described in mammalian peroxisomes, and more than half of them play a role in lipid metabolism. (Mannaerts and Van Veldhoven, 1993; Reddy and Mannaerts, 1994). The other enzymes are involved in catabolism of purines and polyamines, metabolism of amino acids and glyoxylate, and inactivation of reactive oxygen species such as hydrogen peroxide, superoxide anions and epoxides (Mannaerts and Van Veldhoven, 1993). An interesting feature of the peroxisomal lipid-metabolizing enzymes in mammalian cells is enzyme duplication in other cell compartments, such as cytosol, mitochondria and endoplasmic reticulum. (Mannaerts and Van Veldhoven, 1993; Reddy and Mannaerts, 1994). The enzyme content of peroxisomes also varies from species to species, and even from tissue to tissue within the same species (Mannaerts and Van Veldhoven, 1993). In this section, I will only focus on the peroxisomal fatty acid β-oxidation enzymes and the difference between mitochondrial and peroxisomal fatty acid β-oxidation.

In lower eukaryotes, peroxisomes appear to be the only subcellular site of β-oxidation (Tolbert, 1981; Van den Bosch et al., 1992). In animal cells, both peroxisomes and mitochondria are capable of degrading fatty acids via β-oxidation (Lazarow, 1978). The peroxisomal membrane contains two forms of fatty acyl-CoA synthetase: one is most active towards long chain fatty acids (C_{14}-C_{20}) (Shindo and Hashimoto, 1978), and a second one most active towards very long chain fatty acids (>C_{20}) (Singh and Poulos, 1988). The latter one is not found in mitochondria (Singh and Poulos, 1988). No carnitine is required for the entry of the fatty acyl-CoA esters in the peroxisome (Mannaerts et al., 1979).

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The peroxisomal β-oxidation sequence is shown in Figure 4 (Mannaerts and Van Veldhoven, 1993). The first oxidation step is catalyzed by a FAD-containing acyl-CoA oxidase (AOX), which reduces molecular oxygen to hydrogen peroxide that is subsequently decomposed by catalase. The activity of AOX largely determines the flux through the overall peroxisomal β-oxidation (Bronfman et al., 1984). Liver peroxisomes contain three acyl-CoA oxidases (Schepers et al., 1990). The one that is induced during peroxisome proliferation and is related to this research is palmitoyl-CoA oxidase. The second (hydration) and third (dehydrogenation) steps are catalyzed by a single protein, which is therefore named "bifunctional enzyme" (Osumi and Hashimoto, 1979). This protein also displays δ^3-δ^2 enoyl-CoA isomerase activity required for the oxidation of unsaturated fatty acids (Palosaari and Hitnunen, 1990), so it is also called "trifunctional enzyme" or "multifunctional enzyme" by some researchers. The last reaction of β-oxidation is catalyzed by 3-ketoacyl-CoA thiolase.

Despite both peroxisomal and mitochondrial β-oxidations consisting of the same four consecutive reactions, there are important differences between the two systems. First of all, the mitochondrial enzymes and their peroxisomal counterparts are different proteins. Peroxisomal β-oxidation is not directly coupled to an electron-transport chain and an oxidative phosphorylation system (Lazarow and de Duve, 1976) so that the energy that is released in the first oxidation step (H_2O_2 production) is lost as heat. It has therefore been speculated that peroxisomal β-oxidation might be involved in thermogenesis (Kramar et al., 1978). However, in quantitative terms its contribution to thermogenesis appears small (Mannaerts and Van Veldhoven, 1993).

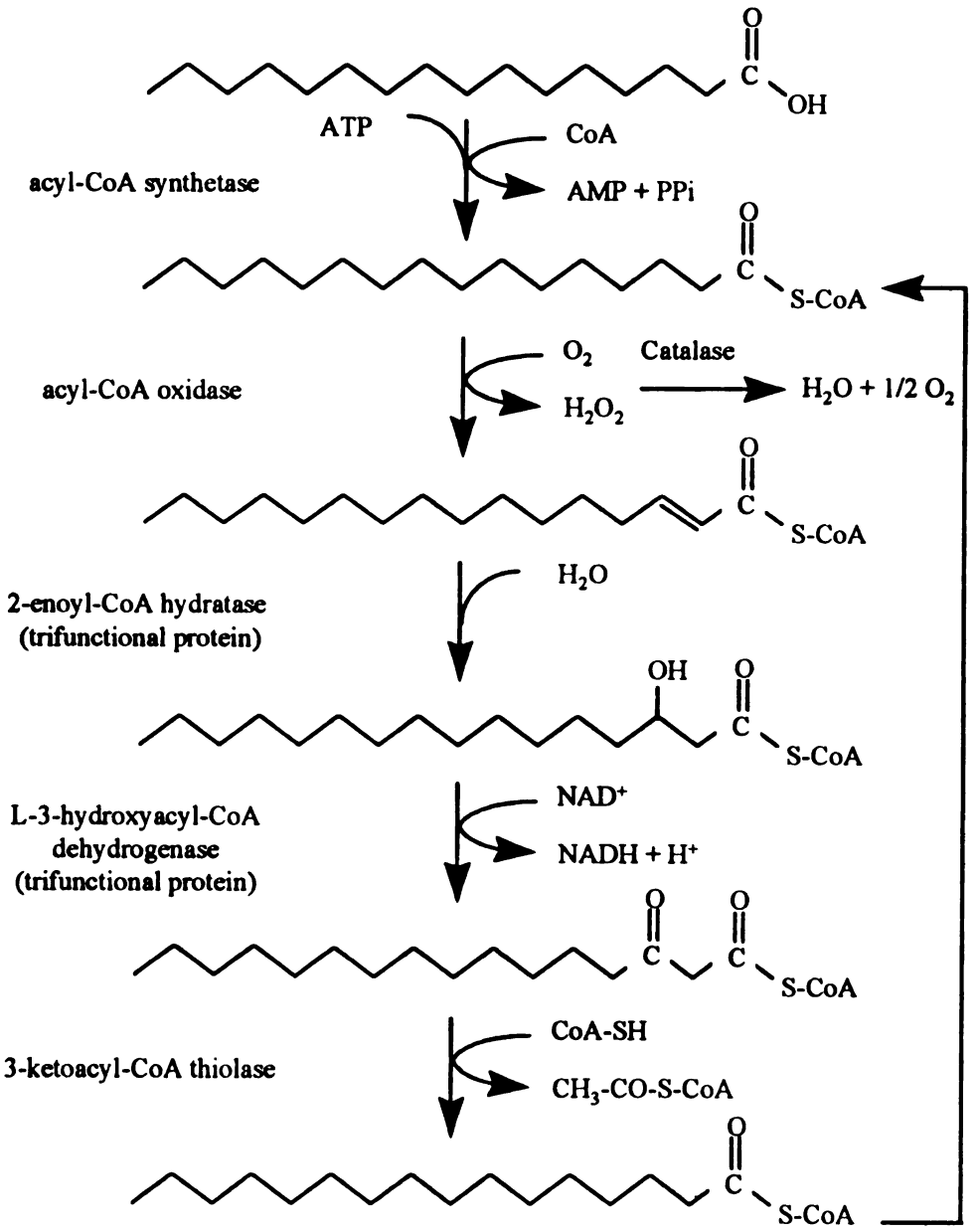

Figure 4. Peroxisomal β-oxidation sequential reactions

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Unlike its mitochondrial counterpart, peroxisomal β-oxidation does not go to completion. Depending on the assay conditions, peroxisomes catalyze only one to five cycles of β-oxidation in *in vivo* studies (Lazarow, 1978; Thomas et al., 1980). This is because fatty acid with a chain length of less than eight carbon atoms are poor substrates or are not substrates for the peroxisomal β-oxidation enzymes (Lazarow, 1978; Osumi and Hashimoto, 1978). Another factor contributing to the premature termination of peroxisomal β-oxidation is the existence of other peroxisomal enzymes that avidly use medium chain acyl-CoA as substrates (Mannaerts and Van Veldhoven, 1993). In addition, it is suggested that because peroxisomal β-oxidation enzymes are not as highly organized and do not channel the β-oxidation intermediates as effectively as mitochondrial enzymes, there is more room for competition by other medium chain acyl-CoA consuming enzymes (Bartlett et al., 1990). The fate of shortened fatty acyl-CoAs is not completely clear yet, but ultimately they have to be either oxidized in the mitochondria or esterified in the peroxisome itself or in the endoplasmic reticulum (Mannaerts and Van Veldhoven, 1993).

Peroxisomal and mitochondrial β-oxidations favor different substrates. While both are able to degrade medium (C_8 to C_{12}) and long (C_{14} to C_{20}) chain fatty acids, short chain ($<C_8$) fatty acids are degraded only in mitochondria, and very long chain ($>C_{20}$) fatty acids are preferred by peroxisomes. It is estimated that more than 90% of long chain fatty acid oxidation is mitochondrial (Mannaerts et al., 1979; Van Veldhoven and Mannaerts, 1985). Long chain fatty acids are by far the most abundant substrate for β-oxidation and they play a major role in fuel homeostasis (Mannaerts and Van Veldhoven, 1993). Since mitochondrial β-oxidation conserves more energy than does peroxisomal β-oxidation, it appears logical that long chain fatty acids are oxidized preferentially by mitochondria.

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Although very long chain fatty acids constitute only a very minor part of the overall fatty acids, their accumulation in peroxisome deficiency disorders is deleterious (Lazarow and Moser, 1989).

Substrates for peroxisomal β-oxidation also include dicarboxylic fatty acids, isoprenoid-derived 2-methyl-branched fatty acids, the side chains of the bile acid intermediates from cholesterol, the side chains of prostaglandins and other eicosanoids, fat-soluble vitamins E and K, and certain xenobiotics (Reddy and Mannaerts, 1994).

Other peroxisomal functions related to lipid metabolism are: α-oxidation of 3-methyl-branched fatty acids (Singh et al., 1993), which are not substrates for β-oxidation, fatty acid elongation and δ^4 desaturation (Voss et al., 1991), ether glycerolipid synthesis (Webber and Hajra, 1993), and cholesterol and dolichol synthesis (Thompson and Krisans, 1991; Stamellos et al., 1992).

6. Peroxisome proliferation

Peroxisome proliferation was first discovered in the livers of rats and mice following the administration of clofibrate, a hypolipidemic drug (Hess et al., 1965). The phenomenon observed is liver enlargement and increase in size and number of peroxisomes. It is primarily seen in the liver and kidney and furthermore is species-specific. Rats and mice are more susceptible to peroxisome proliferators than other organisms, while guinea pigs and primates show little or no responses (Orton et al., 1984).

The agents that cause peroxisome proliferation are designated peroxisome proliferators. They include a broad group of structurally diverse chemicals such as hypolipidemic drugs, phthalate ester plasticizers, solvents (e.g. trichloroethylene),

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Prolonged treatment with peroxisome proliferators often results in hepatocarcinogenesis (Cohen and Grasso, 1981; Reddy and Lalwani, 1983). The basic mechanism of tumor induction is unknown. Since peroxisome proliferators do not damage DNA directly (Warren et al., 1980), they are considered non-genotoxic carcinogens.

Besides morphological changes, peroxisome proliferation is characterized by dramatic increases in the activity of certain peroxisomal enzymes, especially those involved in the β-oxidation process (Lazarow and de Duve, 1976; Hashimoto, 1987). Theses enzymes are represented by acyl-CoA oxidase, bifunctional enzyme and 3-ketoacyl-CoA thiolase (Lock et al., 1989). The mRNA levels of the three enzymes in the rat liver treated with peroxisome proliferators increase coordinately (Reddy et al., 1986) and remain at the induced level as long as peroxisome proliferators are administered (Nemali et al., 1988). Catalase mRNA is also elevated by peroxisome proliferators, but to a much lesser extent (Nemali et al., 1989), while the peroxisomal urate oxidase is not induced by peroxisome proliferators (Usuda et al, 1988).

The levels of certain extraperoxisomal enzymes, in particular members of the microsomal cytochrome P450 (CYP450) family, are also increased in response to peroxisome proliferators (Hardwick et al., 1987). Cytochrome P450 is a gene superfamily consisting of over 200 separate genes and is further subdivided into 36 gene families. These enzymes metabolize (primarily by hydroxylation) a very large number of environmentally derived chemicals, thus facilitating their excretion, and endogenous compounds, including fatty acids, steroids, prostaglandins, leukotrienes and vitamins

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(Gibson et al., 1993). The CYP450 IV family genes appear to be the most susceptible to induction by peroxisome proliferators (Hardwick et al., 1987). These genes show the same tissue- and species-specificities in response to peroxisome proliferators as peroxisomal β-oxidation genes (Lake et al., 1989; Gibson et al., 1993). Based on these observations, it has been proposed that the induction of CYP450 and that of peroxisomal β-oxidation genes by peroxisome proliferators are through a common regulatory pathway (Lake et al., 1984; Green, 1992).

High fat diets cause similar effects as xenobiotic peroxisome proliferators (Neat et al, 1980; Flatmark et al., 1988). Peroxisome proliferation is also observed under conditions characterized by fatty acid overloads, such as after increased dietary fat intake, in times of starvation (Thomas et al., 1989; Orellana et al., 1992) and during uncontrolled diabetes mellitus (Thomas et al, 1989; Hori et al., 1981). As a result of these findings, it has been proposed that increased intrahepatic lipid may be an important factor in the genesis of peroxisome proliferation (Elcombe and Mitchell, 1986). The accumulation of hepatic lipid can occur in a number of ways, and the diverse chemical structures that produce peroxisome proliferation may act at many different loci to perturb lipid metabolism (Lock et al., 1989). According to this theory, peroxisome proliferators are taken up by the liver and initially inhibit fatty acid oxidation by the dual mechanism of inhibition of carnitine acyl transferase in the mitochondrion (Lock et al., 1989) or sequestration of essential CoA by the peroxisome proliferator itself (Bronfman. 1993).Thus cellular medium and long chain fatty acids accumulate in the hepatocyte and microsomal cytochrome P450 IV is substrate induced to maintain cellular lipid homeostasis by fatty acid ω-hydroxylation and subsequent formation of long chain

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dicarboxylic acids (Lock et al., 1989; Sharma et al., 1988). As mitochondria cannot efficiently β-oxidize long chain dicarboxylic acids, the products of microsomal ω-hydroxylation, and these are the preferred substrates for peroxisomal β-oxidation (Mannaerts an Van Veldhoven, 1993), it would seem plausible that this may contribute, at least in part, to the induction of peroxisomal β-oxidation. This theory is supported by a number of reports. First, the induction of CYP450 genes by peroxisome proliferators appears to be a very early event and precedes that of peroxisomal β-oxidation genes as assessed both *in vivo* (Milton et al., 1990) and *in vitro* (Bieri et al, 1991). Second, when rats are administered both clofibrate and protein synthesis inhibitor cycloheximide, CYP450 IVA mRNA is still elevated, but the induction of genes encoding peroxisomal β-oxidation enzymes is not seen under these experimental conditions (Milton et al., 1990).

However, the above substrate overload/perturbation of lipid metabolism mechanism cannot explain peroxisome proliferation by agents that do not inhibit mitochondrial β-oxidation and the rapidity of the transcriptional changes of the responsive genes. An alternative explanation of the peroxisome proliferation phenomenon is the receptor-mediated mechanism (Rao and Reddy, 1987). According to this theory, peroxisome proliferation is triggered at the molecular level by a nuclear receptor which is activated by peroxisome proliferators. The discovery of a peroxisome proliferator activated receptor (Issemann and Green, 1990) opened a new field of research and furthermore has stressed the importance of dietary factors as modulators of gene expression.

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7. Peroxisome Proliferator Activated Receptors (PPARs)

In 1990, a novel nuclear receptor was cloned from mouse liver (Issemann and Green, 1990). Because it was activated by a variety of peroxisome proliferators, it was termed peroxisome proliferator activated receptor (PPAR) and was later renamed mPPARα. So far, three types of PPARs, α, δ (also termed NUC1, FAAR, or PPARβ) and γ, have been identified. The α, β and γ types have been found in *Xenopus* (Dreyer et al., 1992). In mouse and human, an α (Issemann and Green, 1990; Sher et al, 1993), β type (Chen et al, 1993; Amri et al., 1995; Schmidt et al, 1992) and two γ types (Zhu et al, 1993; Tontonoz et al, 1994; Greene et al., 1995; Lambe et al., 1996; Elbrecht et al., 1996; Mukherjee et al., 1997) have been cloned. In rat, only the α form has been isolated (Gottlicher et al., 1992), whereas in hamster a γ form has been isolated (Aperlo et al., 1995). The two PPARγ isoforms of mouse and human, γ1 (Zhu et al., 1993; Greene et al., 1995; Lambe et al., 1996; Elbrecht et al., 1996) and γ2 (Tontonoz et al., 1994; Mukherjee et al., 1997), differing only in their N-terminal 30 amino acids. It has been demonstrated that the mouse PPARγ1 and γ2 derive from the same gene by alternative promoter usage and splicing (Zhu et al., 1995). Differential splicing of the non-coding first exon has also been reported for mPPARα (Gearing et al, 1994).

PPARs are assigned to the nuclear receptor superfamily because they present the characteristic modular structure of nuclear receptors. The PPAR DNA-binding domain is the most conserved region comparing with other nuclear receptors and among different isoforms of PPARs. The P-box of the PPARs is identical to that of TR, VDR, RAR and EAR-1. However, the D-box of PPARs differs from almost all other nuclear receptors,

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since it is only composed of three amino acids instead of five amino acids (Schoonjans et al., 1996). The LBDs of the different PPARs are less conserved comparing with their DBDs, which may indicate that they bind similar, but not identical ligands.

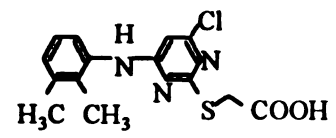
The α, β and γ isoforms of PPAR have their unique tissue specificity and relative abundance. In mouse, PPARα is predominantly expressed in liver, kidney, heart and brown adipose tissue (Issemann and Green, 1990). PPARδ is a ubiquitously expressed type with low levels of expression in liver, kidney and spleen (Amri et al., 1995). The tissue distribution of PPARγ1 is reminiscent of PPARα; while PPARγ2 is predominantly expressed in adipose tissue (Tontonoz et al., 1994).

The expression of PPARs appears to be influenced by environmental conditions. mPPARγ mRNA is induced by ciprofibrate administration (Zhu et al., 1993). It has been reported that rPPARα gene transcription is strongly induced by glucocorticoids and fatty acid treatment of cultured hepatocytes, and the induction is obliterated by insulin (Lemberger et al., 1994; Steineger et al., 1994). Whether rPPARα mRNA is induced by fenofibrate administration is debated (Gebel et al., 1992; Schoojans et al., 1996).

Most exogenous substances known to induce peroxisome proliferation, such as hypolipidemic fibrate drugs, phthalate ester plasticizers and herbicides, have been shown to activate PPARs (Issemann and Green, 1990; Dreyer et al., 1992). A range of fatty acids are also shown to be able to activate PPARs in transient cotransfection assays using a number of cell lines (Gottlicher et al., 1992, Schmidt et al., 1992; Sher et al., 1993; Keller et al., 1993; Issemann et al., 1993; Brun et al., 1996). With respect to the transactivation abilities and potencies of certain compounds, some reports are contradictory with one another. This may be due to the difference in the types of host cells, the combination of

PPAR expression vectors and reporter systems, as well as different transfection and culture conditions used by the researchers. Figure 5 shows the structures of PPAR ligands that are reported to date and some commonly used PPAR activators.

Since the discovery of PPAR, there has been an intense search for their cognate ligands. 15-deoxy-$\Delta^{12,\ 14}$-prostaglandin J_2 (15d-J_2) and a family of its analogue, thiazolidinedione, which are used as antidiabetic agents, have been demonstrated to be ligands for PPARγ (Kliewer et al., 1995; Lehmann et al., 1995, Forman et al., 1995). Leukotriene B_4 (LTB_4) was shown to bind to PPARα with high affinity, and the binding was modestly competed by WY14,643 (Devchand et al., 1996). However, this finding was later questioned by other researchers for the high level of nonspecific binding (Forman, et al., 1997). More recently, a fibrate derivative, GW2331, was found to be a ligand for both PPARα and PPARγ. Furthermore, a number of fatty acids and eicosanoids are shown to be able to efficiently compete with GW2331 for the binding to these two types of PPARs (Kliewer et al., 1997). Forman et al. employed a novel conformational change-based screening strategy and reported that hypolipedimic agents WY14,643, clofibrate, ciprofibrate, long-chain fatty acids, eicosanoids 8-hydroxyeicosatetraenoic acid (8-HETE), 8-hydroxyeicosapentaenoic acid (8-HEPE), as well as a number of prostaglandins, were all ligands for either PPARα or PPARδ, or for both (Forman et al., 1997). However, the significant difference between the potencies of certain compounds to induce conformational change in the binding studies and to activate PPAR in transfection experiments was never addressed in the report, which casts a shadow of doubt on the credibility of this method.



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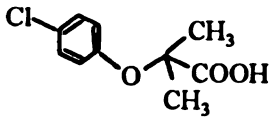

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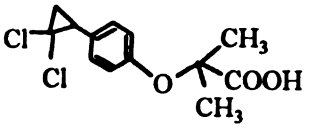

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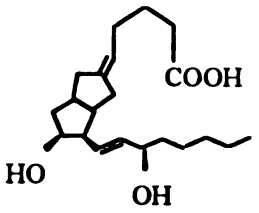
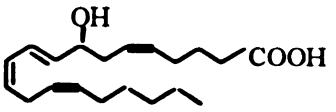
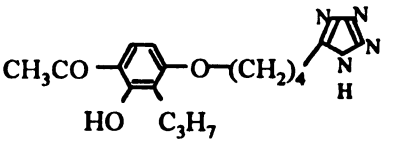


Carba-prostacyclin



8S-HETE



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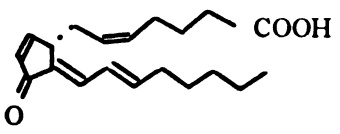
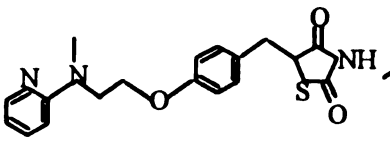


15-deoxy- $\Delta^{12,14}$-PGJ_2



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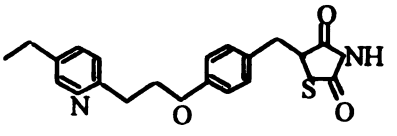
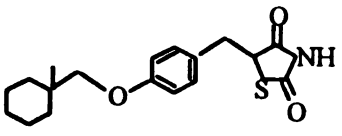
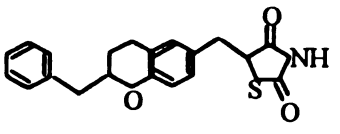


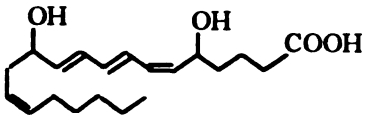
Pioglitazone



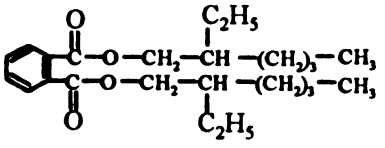
Ciglitazone



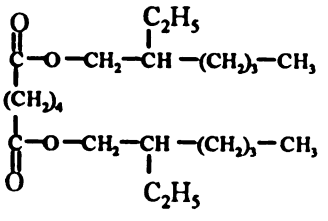
Englitazone



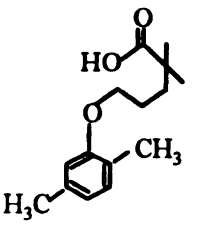
LTB_4



DEHP



DEHA



Gemfibrozil

Figure 5. Chemical structure of some PPAR activators

PPARs recognize a hormone response element, termed peroxisome proliferator response element (PPRE), located in the 5' regulatory regions of the target genes. Most of PPREs characterized are direct repeats of AGGTCA (or TGACCT) motif separated by one intervening nucleotide (DR-1). PPAR bind to PPREs in the form of PPAR/RXR heterodimer (Kliewer et al., 1992). To date, RXR has been the only identified heterodimeric partner of PPAR that is functionally implicated in the peroxisome proliferator signaling pathway.

Besides PPAR/RXR heterodimer, the DR-1 motif is also recognized by RXR homodimer (Mangelsdorf et al., 1991), HNF4 homodimer (Sladek et al., 1990), COUP-TF homodimer (Cooney et al., 1992) and COUP-TF/RXR heterodimer (Kliewer et al., 1992a). This structural degeneracy in target sequence suggests the functional cross-talk between PPAR regulation of gene transcription and other regulatory pathways. It has been reported that COUP-TF inhibits the PPAR/RXR induction of BIEN transcription through the interaction with BIEN-PPRE (Miyata et al., 1993; Marcus et al., 1996).

The cross-talk between PPAR and thyroid hormone receptor (TR) in gene transcriptional regulation has drawn a great deal of attention from a number of research groups. Bogazzi et al. reported that PPAR cotransfection interfered with T_3 control of malic enzyme by forming PPAR/TRβ inactive complex, therefore prevented TR/RXR binding to DNA (Bogazzi et al. 1994). However, their results were contradictory to the reports by other research groups (Juge-Aubry et al., 1995; Chu et al., 1995). Juge-Aubry et al. showed that PPARα interfered with T_3-regulated gene transcription by forming heterodimers with RXRα. The interaction was not due to competition for DNA binding and was independent of PPAR/TR heterodimerization (Juge-Aubry et al., 1995). From

another perspective, Chu et al. demonstrated that TRβ1 interfered with the transcription of bifunctional enzyme by titrating away RXR, but not PPARα, from the PPAR/RXR complex (Chu et al., 1995).

The genes regulated by PPAR include those involved in the peroxisomal β-oxidation pathway: acyl-CoA oxidase (AOX), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (bifunctional enzyme) and 3-ketoacyl-CoA thiolase. PPREs have been localized in the 5' regulatory regions of AOX and bifunctional enzyme genes (Tugwood et al., 1992; Zhang et al., 1992; 1993). Some genes encoding extra-peroxisomal enzymes and proteins are also regulated by PPAR. Two distinct functional PPREs in the promoter of the CYP4A6 gene, which encodes the microsomal cytochrome P450 fatty acid ω-hydoxylase, have been identified (Muerhoff et al., 1992). Other PPRE-containing genes include genes encoding acyl-CoA synthetase (Schoonjans et al., 1995), liver ketogenic HMG-CoA synthase (Rodriguez et al., 1994), mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD) (Gulick et al., 1994), the cytosolic liver fatty acid binding protein (FABP) (Issemann et al., 1992; Kaikkaus et al., 1993), adipocyte fatty acid binding protein P2 (aP2) (Tontonoz et al., 1994), malic enzyme (Castelein et al., 1994) and phosphoenolpyruvate carboxykinase (PEPCK) (Tontonoz et al., 1995).

PPAR is involved not only in peroxisomal and intracellular lipid metabolism, but is also an important regulator of extracellular lipid metabolism and cellular uptake of fatty acids. Apo A-I and apo A-II are the major protein constituents of HDL. Lipoprotein lipase (LPL) hydrolyzes the TG moiety of chylomicrons and VLDL particles. PPREs have been found in the promoters of all these genes (Widom et al., 1991; Vu-Dac et al., 1994; Ladias et al., 1992; Cardot et al., 1993; Schoonjans et al., 1996). Fatty acid transporter protein

(FATP) and fatty acid transporter (FAT) are also regulated by PPAR (Amri et al., 1995; Schoonjans et al., 1996).

In cells, different PPAR isoforms may exert unique biological functions. When the PPARα LBD is disrupted by homologous recombination, mice homozygous for the mutation lack expression of mPPARα and yet are viable, fertile and exhibit no detectable gross phenotypic defects. Remarkably, the knockout mice lose the pleiotropic responses, such as hepatomegaly, peroxisome proliferation, and transcriptional induction of AOX, BIEN, CYP4A1, CYP4A3 and FABP, when challenged with peroxisome proliferators clofibrate and WY14,643 (Lee et al., 1995). This has clearly demonstrated that PPARα is the major isoform required for mediating actions of peroxisome proliferators on peroxisomal β-oxidative gene transcription.

The other two isoforms of PPARs, especially PPARγ, are thought to play crucial roles in adipocyte differentiation. During embryogenesis, an early expression of mPPARδ clearly preceding the onset of mPPARα and mPPARγ expression (Kliewer et al, 1994; Amri et al., 1995). Fibroblast cell line 3T3-C2 is unresponsive to FA administration. However, when mPPARδ are cotransfected, the cells become responsive to FA, marked by the induction of two adipocyte differentiation marker genes, adipocyte lipid binding protein (ALBP) and fatty acid transporter (FAT) (Amri et al., 1995). Forced expression of mPPARγ, but not mPPARα, is sufficient to convert fibroblast cell lines into differentiation-competent preadipocytes (Tontonoz et al., 1994b). It is proposed that the interaction of PPARγ and CCAATT enhancer binding proteins (C/EBP) is the initial trigger for the adipogenic program (Tontonoz et al., 1994b; Hu et al., 1995; Wu et al.,

1995; Brun et al., 1996). This hypothesis is supported by the finding that prostaglandin J_2, a potent inducer of adipocyte differentiation, is the natural ligand for PPARγ (Lehmann et al., 1995; Forman et al., 1995).

Despite the substantial amount of evidence for a direct involvement of PPARs in the generation of peroxisome proliferation, the receptor-mediated mechanism can not fully explain the complexity of this phenomenon. For example, it has been reported that a number of agents that induce peroxisome proliferation, such as DHEA and DHEA sulfate, fails to activate mPPAR (Issemann and Green, 1990) and rPPAR (Gottlicher et al., 1992). This raises the possibility of multiple signaling mechanisms for the induction of the fatty acid β-oxidation system by peroxisome proliferators and fatty acids.

8. Rationale for current studies

Fat accounts for 39% of energy in the average American diet (Willett, 1994). Dietary fats have pronounced effects on gene expression leading to changes in metabolism, cell growth and differentiation (Jump et al., 1994; Amri et al., 1991; 1994). Furthermore, a number of chronic diseases or risk factors for chronic diseases, including insulin-resistant diabetes (Storlien et al., 1987; McGarry 1992), coronary artery disease (Ascherio et al., 1995; Clarke and Jump, 1994), obesity (Pan et al., 1994) and breast, colon, pancreas and prostate cancers (Cave, 1991), have been linked to the type and amount of fat we ingest. Dietary fats appear to influence the onset and progression of these diseases at two levels: 1) alteration in membrane phospholipid composition, which results in changes in hormone signaling (Cave, 1991; Clandinin et al., 1991); and 2) direct control of the nuclear events that govern gene transcription (Armstrong et al., 1991;

Clarke et al., 1990, Clarke and Jump, 1994). These two levels of control interact with each other and enable fatty acids to exert both rapid and long-term adaptive modulation of gene expression (Clarke and Jump, 1994).

Our laboratory has been interested in dietary PUFA regulation of hepatic lipogenic gene expression. Using a combination of in vivo and in vitro approaches, we have found that dietary PUFA specifically and coordinately suppress the mRNAs encoding L-PK, FAS, ME and S14 (Jump et al., 1994). To further understand the molecular mechanism by which PUFA suppress these mRNAs, S14 gene has been used as a model system because of its well characterized regulatory structure, which is not available at the same level for other genes. Before I joined this research group, they already demonstrated that PUFA inhibit S14 gene expression at the transcriptional level. The cis-linked PUFA responsive elements have been localized within the S14 proximal promoter region (-80 to -220 bp). This region also contains cis-acting elements that potentiate T_3 activation of S14 gene transcription (Jump et al., 1993).

My research project was designed to investigate the molecular mediator of PUFA action on S14 gene transcription. Initially we speculated PUFA might regulate S14 gene transcription via PPAR. Our speculation arose from the observations that 1) both fatty acids and peroxisome proliferators cause peroxisome proliferation in rodent liver (Flatmark et al., 1988; Rustan et al., 1992; Mikkelsen et al., 1993) and 2) fatty acids have been shown to activate PPAR in *in vitro* transfection studies (Gottlicher et al., 1992; Schmidt et al., 1992). Before leukotriene B_4 was identified as a natural ligand for PPARα (Devchand et al., 1996), a well accepted surmise was that fatty acid might be the natural ligand for this orphan receptor (Green and Whali, 1994).

If PUFA exert their effects on S14 gene transcription via the action of PPAR, we would expect to see 1) a similar inhibitory effect on S14 gene by peroxisome proliferators, the bona fide activator of PPAR, and 2) PUFA and peroxisome proliferators target the same *cis*-regulatory element within the S14 promoter region. Therefore I started my research project by examining the effect of a potent peroxisome proliferator, WY14,643, on S14 gene expression in *in vitro* studies. The preliminary studies showed that WY14,643 suppressed both S14 mRNA and S14CAT reporter gene in cultured primary hepatocytes. Subsequent promoter deletion analysis showed that both WY14,643 and PPARα targeted the far upstream TRR, instead of the PUFA-RR within the S14 proximal promoter.

My thesis was designed to answer the following questions: 1) Do PUFA and peroxisome proliferators regulate hepatic S14 gene expression through the same pathway? 2) What is the molecular mechanism that PPAR inhibit S14 gene expression? 3) Is PPAR involved in PUFA regulation of hepatic S14 gene transcription? and 4) Under what physiological conditions does PPARα participate in PUFA regulation of S14 gene transcription?

Under the guidance of Dr. Jump, I have focused on these aims and provided answers to the above questions. Some answers are relatively clear and some can only be used as preliminary data for further studies. I hope my work will prove to be helpful in understanding the complex molecular mechanism of lipogenic regulation and eventually contribute to a solution for some dietary fat- and peroxisome proliferator-linked diseases.

Chapter 2. A Potent Peroxisome Proliferator Wy14,643 Inhibits S14 Gene Transcription through Activation of PPARα

Introduction

Highly unsaturated n-3 dietary polyunsaturated fatty acids (PUFA) lower serum triglycerides by inhibiting hepatic VLDL production (Phillipson et al., 1985; Nestel et al., 1984; Rustan et al., 1992). This is accomplished, at least in part, by inhibiting both lipogenesis and triglyceride synthesis. The suppressive effect of PUFA on hepatic lipogenesis is due to an inhibition of the activity of several key enzymes involved in lipogenesis and glycolysis (Clarke and Jump, 1994). Further studies have shown that PUFA suppress the hepatic level of fatty acid synthase, pyruvate kinase, stearoyl CoA desaturase-1 and the S14 protein by inhibiting the transcription of genes encoding these proteins (Jump et al., 1993, 1994; 1995; Liimatta et al., 1994; Landschulz et al., 1994). In the case of the S14 protein and pyruvate kinase, PUFA-regulated cis-acting elements (PUFA-RE) have been localized to the proximal promoter regions of these genes (Jump et al., 1993; Liimatta et al., 1994).

Peroxisomal proliferators are a chemically diverse group of compounds that also lower serum triglycerides (Green, 1992). The mechanism for this effect appears to involve induction of transcription of genes encoding several enzymes involved in peroxisomal β-oxidation. Recent studies have shown that peroxisome proliferators augment transcription of acyl CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase and CYP4A6 through a peroxisome proliferator response element (PPRE) that contains a direct repeat of TGACCT motif, separated by one nucleotide (DR-1) (Osumi et al., 1991;

Tugwood et al., 1993; Muerhoff and Johnson, 1992). Peroxisome proliferators activate a nuclear transcription factor identified as peroxisome proliferator activated receptor, i.e. PPAR, through an unknown mechanism (Issemann and Green, 1990; Green, 1992). PPAR is a member of the steroid/thyroid receptor superfamily and binds PPREs in association with retinoid X receptor (RXR) (Keller et al., 1992; Kliewer et al., 1992; Gearing et al., 1993). Several PPAR isoforms have been identified in humans, rodents and Xenopus (Issemann and Green, 1990; Gottlicher et al., 1992; Keller et al., 1992; Schmidt et al., 1992; Zhu et al., 1993).

Since long chain PUFA (> 20 carbons) also induce peroxisomal enzymes (Neat et al., 1980; Flatmark et al., 1988), it seemed possible that PUFA regulation of lipogenic gene transcription might involve PPAR. To determine whether PPAR was involved in the control of S14 gene transcription, I examined the effects of PPARα and the potent peroxisomal proliferator, i.e. Wy14,543, on the regulation of S14 gene transcription in cultured hepatocytes.

Materials and Methods

Plasmids: Reporter plasmids S14CAT124, S14CAT149 and RSVCAT have been described previously (Jump et al, 1993). S14CAT124 and S14CAT149 contain S14 genomic DNA that have 3' ends point at +19 bp and 5' ends point at -4315 and -290 bp, respectively, from the S14 gene transcription start site. RSVCAT (obtained from S. Conrad, Cellular and Molecular Biology Program, Michigan State University) contains the enhancer and promoter sequence from Rous sarcoma virus (RSV) genome. These regulatory elements were inserted in the proper orientation upstream of the bacterial

chloramphenicol acetyl transferase (CAT) gene in the pCAT (An) plasmid (obtained from H. Towle, University of Minnesota), which also contains 2 SV40 polyadenylation signals. TKCAT223 contains the rat acyl CoA-oxidase (AOX)-PPRE. TKCAT223 was constructed by PCR amplification of the region between -1198 and -463 bp upstream from the rat AOX gene (Miyazawa et al., 1987) using rat genomic DNA as template and oligonucleotide primers (sense: 5'-ATATGGATCCCCAGTAGAACCTTGTTCAGG [DJ94] and antisense: 5'-ATATAAGCTTCAGGGTCTCGGGCGGAGTGAAG [DJ95]) (synthesized at the Michigan State University Macromolecular Structure Facility). After amplification, the 735 bp fragment was gel purified and inserted upstream from the TK promoter. The expression vector MLVTRβ1 (obtained from V. Mahdavi, Boston, MA) contains the murine leukemia virus promoter and the rat liver thyroid hormone receptor β1 cDNA. The expression vector for RXRα (obtained from P. Chambon) contains cDNA encoding the mouse retinoid X receptor (RXR).

Hepatocyte Culture: Primary hepatocytes were prepared using the modified collagenase perfusion method (Berry and Friend, 1969; Jacoby et al., 1989). Male Sprague-Dawley rats (150-350g) maintained on Teklad chow were fasted for 24 hours prior to hepatocyte preparation. Rats were sacrificed and liver perfused with oxygenated perfusion buffer I (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM EGTA, pH 7.4) and buffer II (66.7 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 4.8 mM $CaCl_2$, pH 7.6) supplemented with 1% fatty acid free albumin (Boehringer-Mannheimm) and 0.01-0.03% collagenase-B (Boehringer-Mannheim), depending on the age of the rats. The liver was excised and rinsed with 25 ml plating media (Williams E [Life Technologies] supplemented with 25 mM glucose, 26 mM $NaHCO_3$, 0.2 mM HEPES, 1.2% [v/v]

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Penicillin-Streptomycin solution [Sigma], 10 nM dexamethasone, 1 μM insulin, 10% heat inactivated fetal calf serum [Intergen Company], pH 7.2) for three times. The liver was dispersed with a metal comb. The tissue suspension was filtered through 100-gauze and 153 gauze filters. The flow-through was centrifuged at 10 × g for 10 minutes, resuspended and layered over Percoll cushion (50% [v/v] plating media, 50% [v/v] Percoll stock. Percoll stock is 89% [v/v] Percoll [Pharmacia], 10% 10 × PBS stock, 10 mM HEPES, pH 7.4) and centrifuged at 270 × g for 10 minutes. 10 × PBS stock consisted of 1.37 M NaCl, 27 mM KCl, 0.1 M Na_2HPO_4, 17.6 mM KH_2HPO_4, pH 7.4. The cells at the bottom of the Percoll cushion were recovered and resuspended in plating medium and plated at 3×10^6 cells per 60-mm plastic tissue culture dish (Primaria) for transfection studies, and at 10^7 cells per 100-mm dish for RNA analysis.

Transfection of hepatocytes: The hepatocytes were transfected using Lipofectin (Life Technologies). The formation of liposome-DNA complexes was carried out by mixing 1-5 μg of plasmid DNA with Lipofectin to give a ratio of 1: 6.6 (wt/wt) in 4 ml of medium. This mixture was added directly to each dish of cells that had been washed with 1 × PBS. After treatment with the DNA-liposome mixture for 12 hours in a 5% CO_2 incubator (Forma Scientific, Marietta, OH), medium was replaced with serum-free experimental medium. Hepatocytes were treated with 100 μM Wy14,643 (Chemsyn Science Laboratories, Lenexa, KS). Wy14,643 was dissolved in Me_2SO, which was used as control for Wy14,643 treatment. The medium was changed every 24 hours. After 48 hours of incubation in experimental medium, the cells were harvested for CAT analysis.

CAT analysis: CAT activity analysis was performed as mentioned previously (Jump et al., 1993). Protein concentration of harvested cells were determined using the

Biorad Protein Assay (Bradford, 1976) in order to normalize CAT activities. Harvested cells were heated to 70° C for 10 minutes to inactivate proteases and deacetylases. Reactions contained 120 µl cells suspended in 250 mM Tris-Cl, pH 7.5, 2.5 µl 10 mM butyryl-CoA and 0.1 µCi of ^{14}C-chloramphenicol (NEN™). Reactions were incubated at 37° C for 2 hours and terminated with 300 µl mixed xylenes (Aldrich). Tubes were vortexed vigorously for 1 minutes and centrifuged for 10 minutes in the microcentrifuge. The upper phase (mixed xylenes and butyrylated chloramphenicol) was removed to a new microfuge tube, and extracted with 200 µl of Tris-Cl (250 mM, pH 7.5). The process was repeated once and the upper phase was quantitatively removed to a scintillation vial. After addition of 5 ml of Safety-Solve (Research Products International Corp., Mount Prospect, IL), vials were counted in a liquid scintillation counter (Beckman LS 3150P). Values of counts were compared against a standard curve generated using purified CAT enzyme (Pharmacia) and were expressed as CAT units (cpm/100 µg protein/hour).

RNA extraction from hepatocytes: Total RNA was extracted from hepatocytes by the guanidium thiocyanate procedure (Chirgwin et al., 1979). The cells were washed with PBS and frozen at -80° C. The cells were collected by scraping with a rubber policeman in 2 ml 4 M guanidium thiocyanate supplemented with 12.6 mM sodium citrate, 17 mM sodium-sarcosyl, 0.1 M β-mercaptoethanol. The cell suspension was homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) in 3 ml chloropane (1:1 [v/v] distilled phenol and chloroform) in a 15 ml Corex tube. After centrifugation, the upper phase was taken and homogenized with a Polytron in 4 ml chloroform: Isoamoyl alcohol (24:1 [v/v]). The suspension was centrifuged and the upper phase was transferred to a clean Corex tube. 200 µl Buffer III (2 M sodium acetate, pH 5.0) and 4 ml isopropanol

were added and the tube was placed at -20° C overnight. The next day, the pellet was collected by centrifugation, resuspended in 500 µl Buffer II (7 M guanidine-HCl, 20 mM sodium acetate, 1 mM dithiothreitol, 10 mM iodoacetamide, 1 mM EDTA, pH 8.0). 300 µl of ethanol and 50 µl of Buffer III were added and the tube was placed at -20° C for at least 2 hours. The pellet was collected by centrifugation and sequentially washed with 500 µl Buffer IV (3 M sodium acetate, 10 mM iodoacetamide, pH 5.0), 500 µl Buffer V (33 mM sodium acetate, 67% [v/v] ethanol), 500 µl ethanol. The pellet was dried and suspended in 250 µl TE-8 and quantified for RNA concentration on a spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH).

RNA Analysis: Levels of RNA were measured by dot blot or Northern blot hybridization using the standard method (Sambrook et al., 1989). Probes for specific mRNAs were labeled with ^{32}P using Random Primed DNA labeling Kit (Boehinger Mamheim). pTZ18R contains cDNA encoding rat acyl CoA oxidase (obtained from T. Osumi, Himeji Inst, Japan [Miyazawa et al., 1989]). pS14exoPEII-8 contains genomic S14 sequences representing +23 to +483 bp and the 5' exon of the S14 gene. pFAS-17 (obtained from S. D. Clarke, Colorado State University) represents FAS-17 cDNA cloned originally by Nepokroeff et al. (1984). pPK (obtained from A. Kahn, INSERM, France) contains cDNA encoding the liver-type pyruvate kinase. pβ-actin (obtained from L. Kedes, Stanford University) contains cDNA encoding β-actin. Following hybridization, blots were washed, dried and exposed to X-ray film. For some autoradiographs, relative levels of hybridization were quantified using videodensitometry.

Determination of Cell Viability: Viability of hepatocytes after Wy14,643 was determined by measuring the leakage of lactate dehydrogenase (LDH) from hepatocytes.

Hepatocytes were treated with Wy14,643 as described above. After the 48 hour treatment, media were collected and cell debris was removed by centrifugation (12,000 × g, 5 minutes). Cells were covered by 500 µl buffer (250 mM Tris-HCl, pH 7.5) and frozen at -80° C. Cells were thawed and scraped, and then subjected to three freeze/thaw cycles. The cell homogenate and the cell culture media were assayed for LDH activity using an LDH kit (Sigma). Total protein within the homogenate was determined using the Bradford assay (Bio-Rad). Total barbituric acid reactive substance was assayed in the medium (Kosugi et al., 1989; Hostmark and Lystad, 1992).

Gel Mobility Shift Assays: The gel mobility shift assay was performed essentially as described by Garner and Revzin (1981) and by Fried and Crothers (1981). The AOX-PPRE was synthesized using oligonucleotides: 5'-GATCCTCCCGAACGTGACCTTT-GTCCTGGTCCA and 5'-AGCTTGGACCAGGACAAAGGTCACGTTCGGGAG, annealed, and end-labeled with ^{32}P using T_4-polynucleotide kinase. Nuclear receptors were synthesized in vitro by programming the TNT transcription/translation system (Promega) with 1µg of expression plasmid containing the cDNAs encoding the receptors. DNA-protein complexes were formed by incubating end-labeled DNA (1-10 fmol) for 20 min at room temperature in a reaction mixture containing 4 ml unprogrammed cell lysate or 2 ml of RXRα- and 2 ml of mPPARα-programmed cell lysate in buffer [25 mM-Tris/HCl (pH 7.5), 10% glycerol, 40 mM-KCl, 0.1 mM-EDTA, 1 mM-dithiothreitol (DTT), 0.5 mM-$MgCl_2$ and 2 mg of poly[d(I-C)] (Boehringer-Mannheim)] (MacDougald and Jump, 1991). Unlabeled AOX-PPRE and S14 proximal promoter elements were used as competitors and were added prior to the addition of labeled probe. After the binding reaction, 5 ml of buffer containing 0.16% bromophenol blue and 0.16% xylene cyanol was added to the

DNA-protein complex prior to loading on to an 8% polyacrylamide gel (acrylamide/bisacrylamide 75:1, w/w) with 0.25 × TBE (1 × TBE = 89 mM-Tris, 89 mM borate, 2.5 mM EDTA, pH 8.3) as electrophoresis buffer. After electrophoresis at 350 V for 90 min, the gels were dried, exposed to X-ray film at -80 °C with intensifying screens.

Results

Wy14,643 and mPPARα Stimulate Acyl-CoA Oxidase Gene Expression in Cultured Primary Hepatocytes. The effect of a potent peroxisome proliferator, Wy14,643 (Issemann and Green, 1990), was examined in cultured primary hepatocytes. Hepatocytes were treated with either the vehicle (Me_2SO) or increasing concentration (50 - 300 μM) Wy14,643. Peroxisomal acyl-CoA oxidase (AOX) gene was used as a marker of peroxisome proliferation (Lock et al., 1989; Tugwood et al., 1992). mRNA encoding acyl-CoA oxidase (AOX) gene was measured by dot blot hybridization analysis and quantified with a video densitometer. The results are shown in Figure 6. In the presence of Me_2SO, relatively low level of $mRNA_{AOX}$ was detected. Addition of increasing concentration of Wy14,643 led to an induction of $mRNA_{AOX}$ in a dose dependent manner. The highest level of $mRNA_{AOX}$ was seen with 300 μM Wy14,643. The effects of Wy14,643 at higher concentrations (up to 1 mM) were also examined (data not shown). It appeared that Wy14,643 at concentrations higher than 300 μM exhibited toxic effect on hepatocytes, as indicated by substantial portion of the cells detaching from the culture dish.

The pleiotropic effects of peroxisome proliferators are mediated, at least in part, by PPAR (Issemann and Green, 1990; Green, 1992; Kliewer et al., 1992). To determine the

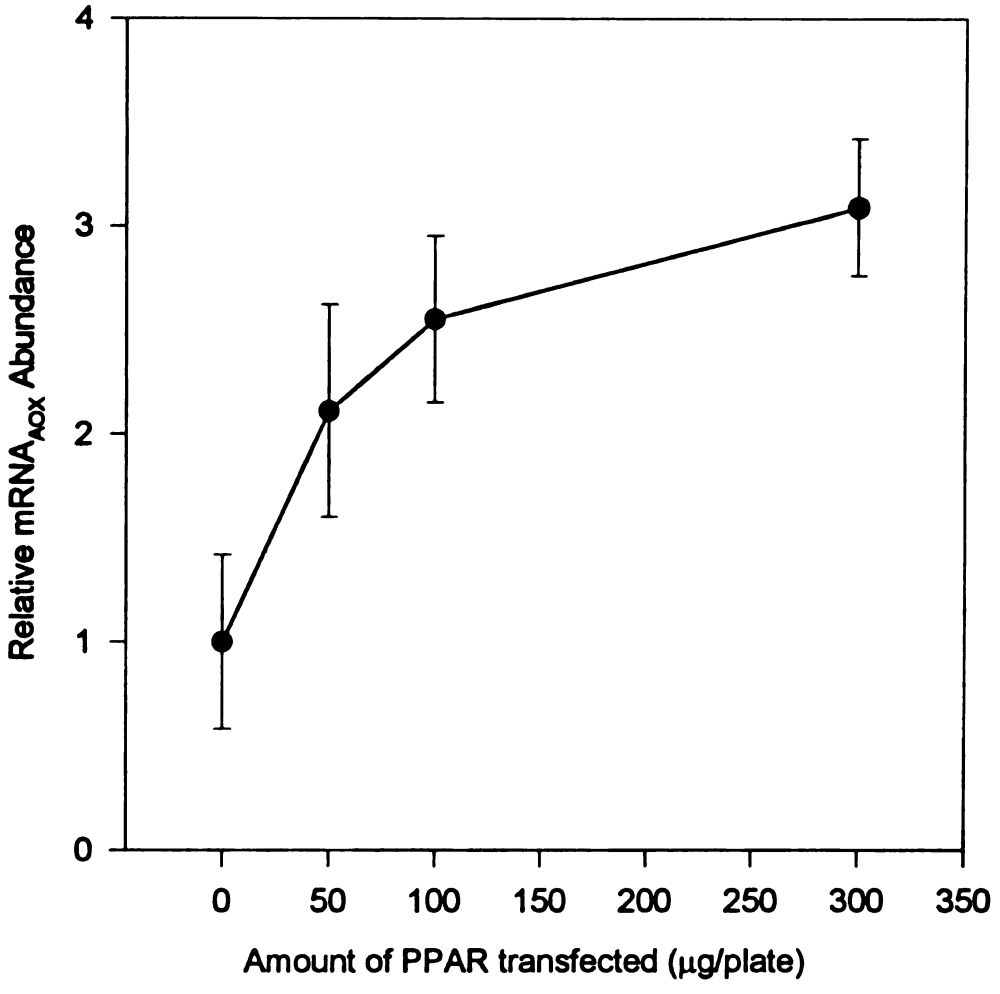


Figure 6. Dose response of hepatocyte mRNA$_{AOX}$ to Wy14,643

Primary rat hepatocytes prepared by the collagenase perfusion were plated into Primaria tissue culture dishes in the presence of 1 μM T_3, 1 μM insulin and 10 nM dexamethasone. The cells were maintained in media containing Me_2SO or different concentrations of Wy14,643 for 48 hours. Total RNA was prepared from the hepatocytes and analyzed by dot blot analysis for mRNA coding for AOX. The results are quantified by videodensitometry. N=3.

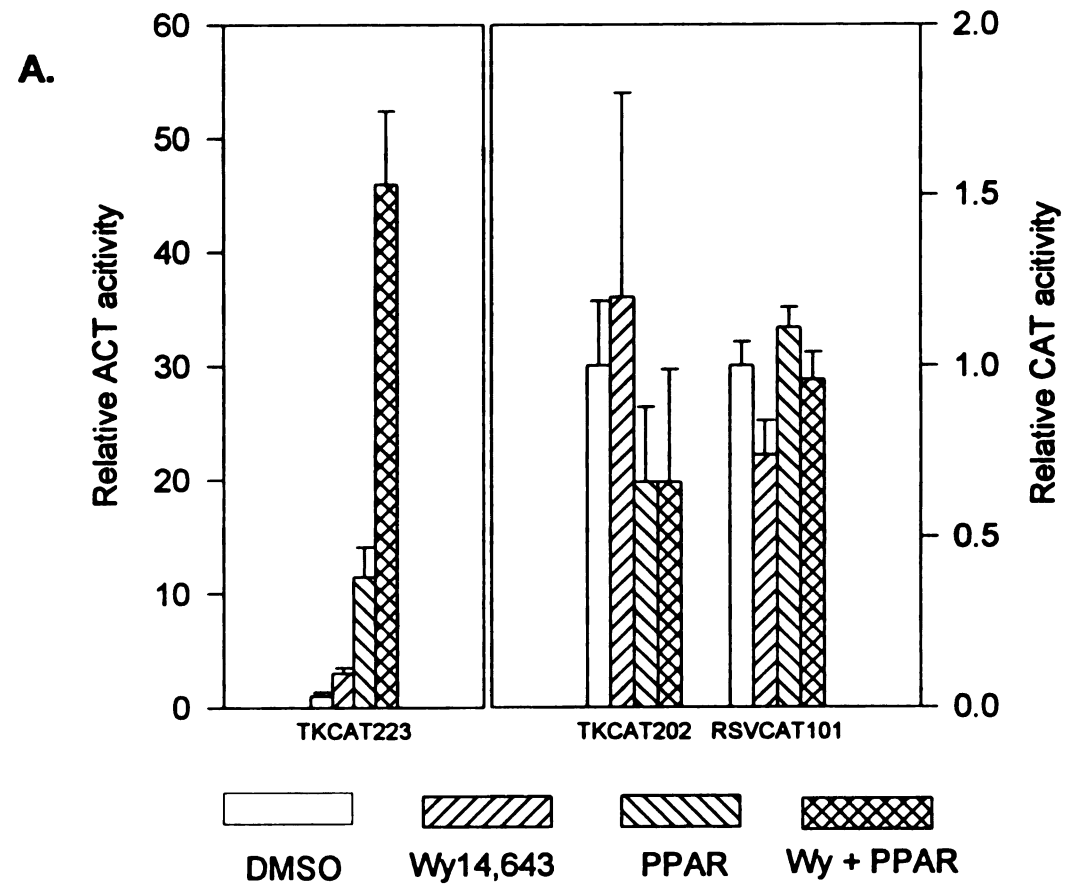
involvement of PPAR in the induction of $mRNA_{AOX}$ in hepatocytes, transfection experiments were conducted. Hepatocytes were transfected with reporter plasmid TKCAT223, which contains the PPRE from AOX (Miyazawa et al., 1987) inserted upstream of the minimal TK promoter, in the presence or absence of an expression vector containing the full-length cDNA encoding mouse PPARα. The α isoform was chosen because this is the dominant type of PPAR detected in rat and mouse liver (Issemann and Green, 1990). The cells were treated with either Me_2SO or Wy14,643. After 48 hours the cells were harvested and assayed for CAT activity (Figure 7).

The results show that in the absence of cotransfected PPAR and Wy14,643, TKCAT223 exhibited only weak CAT activity. Addition of 100 μM Wy14,643 caused a 3-fold induction of CAT activity. Cotransfection of PPAR induced the CAT activity to 12-fold. When both Wy14,643 and PPAR are present in the hepatocytes, a maximum CAT activity (45-fold) is observed. A similar pattern of induction was seen with RSVCAT128, which contains the AOX-PPRE and the RSV minimal promoter, albeit with weaker fold induction (2-4 fold, data not shown). This experiment demonstrates that Wy14,643 is an effective activator of PPARα under the conditions of cultured primary hepatocytes. The observation is consistent with the reports from other research groups (Issemann and Green, 1990; Green, 1992; Tugwood et al., 1993; Muerhoff et al., 1992; Keller et al., 1992; Gottlicher et al., 1992; Zhang et al., 1993; Gearing et al., 1993). The induction by Wy14,643 and PPAR is specific to AOX-PPRE because TKCAT202, which contains only the TK minimal promoter, was not induced by either Wy14,643 treatment, PPAR cotransfection, or the combination of both. The CAT activity of RSVCAT101, which

Figure 7. Wy14,643 and PPAR target AOX-PPRE

A. Hepatocytes were cotransfected with reporter genes (2 μg/well) and were treated with either DMSO or 100 μM Wy14,643. Half of the hepatocytes cultures were also cotransfected with 0.2 μg of pSG5-PPARα, then treated with T_3 and either DMSO or Wy14,643. CAT activity for DMSO treated cells transfected with TKCAT223, TKCAT202, and RSVCAT101 was 73.7 ± 22.0, 86.2 ± 16.8 and 53972 ± 3271 CAT Units, respectively. Relative CAT Activity was normalized against CAT activity seen in DMSO treated cells (Mean ± SE, $N \geq 6$).

B. Schematic representation of the structures of the plasmids used in this study.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Relative CAT acitivity
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20
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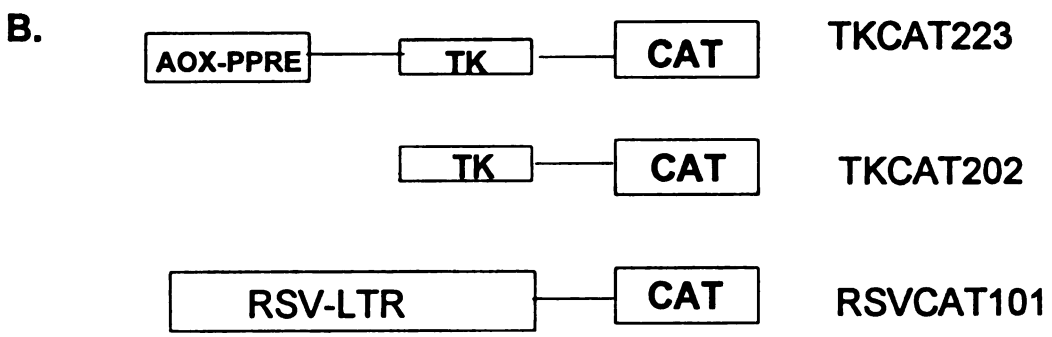
Wy + PPAR


B.
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The amount of cotransfected PPARα was titrated to maximize the PPAR effect. This was examined both in the absence and presence of Wy14,643. The results are shown in Figure 8. In the absence of Wy14,643, minimal amount of PPARα used in the study (0.1 μg) induced CAT activity by 24 fold. Increasing amount of PPAR above 0.1 μg did not substantially induce the transcriptional activity from the heterologous promoter of AOX-PPRE and TK minimal promoter. When Wy14,643 was present, the activity of TKCAT223 was further increased and became more dependent on the amount of cotransfected PPAR until 0.5 μg/plate PPAR was used. In the presence of Wy14,643 and when more than 0.5 μg/plate PPAR was used, TKCAT223 activity showed a moderate decline and became comparable to that when 0.1 μg/plate PPAR was used. Substitution of an equivalent amount of the empty vector, i.e., pSG5, for pSG5-PPARα did not significantly affect TKCAT223 activity (data not shown).

Wy14,643 Suppresses FAS, PK and S14 mRNAs in Cultured Primary Hepatocytes. In order to evaluate the effects of peroxisome proliferator on hepatic lipogenic gene expression, I tested the effect of Wy14,643 on the level of mRNA encoding FAS, PK and S14 protein in cultured hepatocytes. Cultured primary hepatocytes were treated for 48 hrs with T_3 and either vehicle (Me_2SO) or Wy14,643. T_3 was used in both the control and the experimental group because it is required to induce S14 gene transcription in rat hepatocytes (Jump et al., 1993). mRNA encoding the hepatic S14 protein was measured by Northern blot analysis and is shown in Figure 9. In the presence

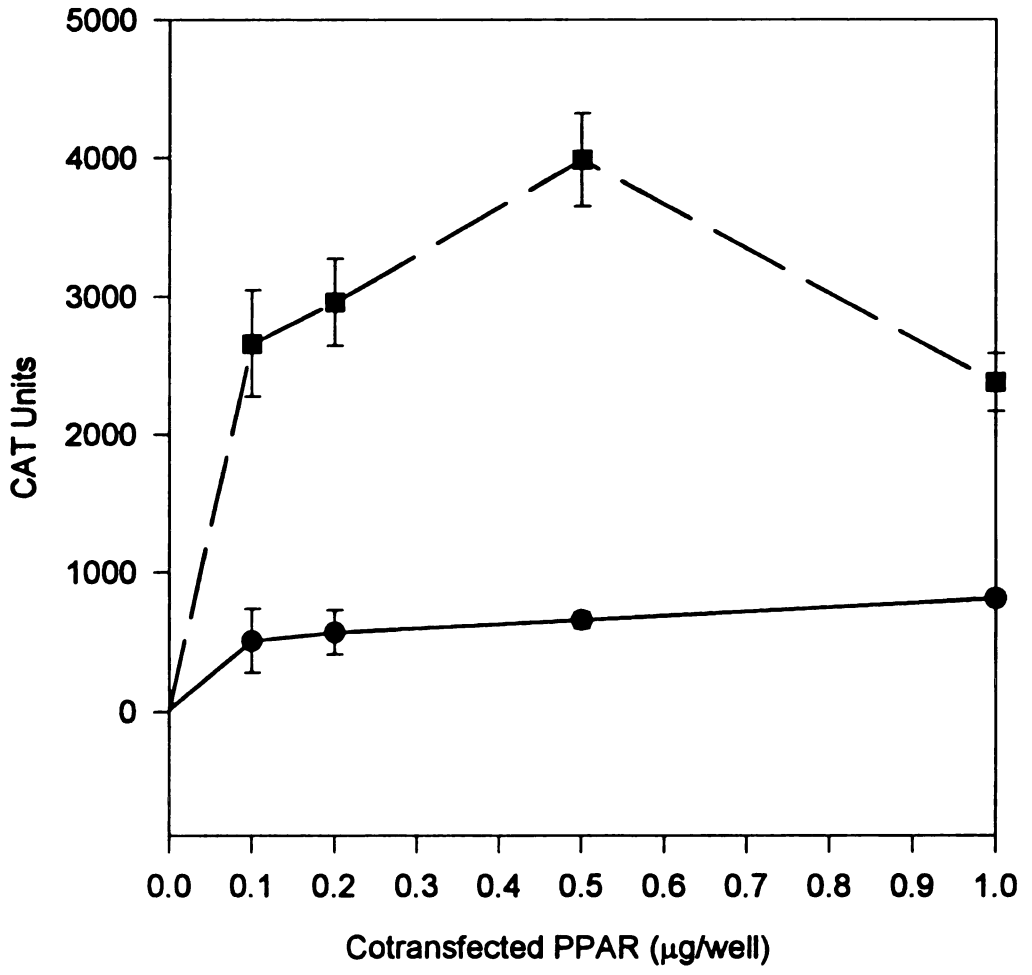


Figure 8. Dose response of TKCAT223 to cotransfected PPAR

Hepatocytes were cotransfected with TKCAT223 (2 μg) and increasing amount of pSG5-PPAR (0 - 1.0 μg) and were treated with either DMSO (circles) or 100 μM Wy14,643 (squares). After 48 hours, cells were harvested for CAT activity(Mean ± SE, N ≥ 6).

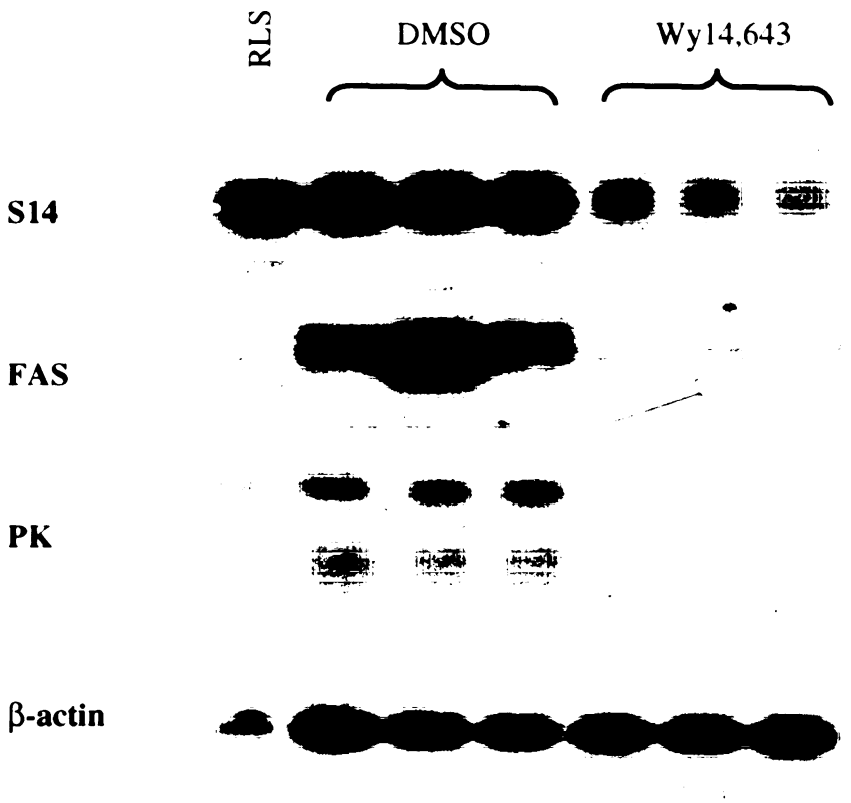


Figure 9. Wy14,643 suppresses hepatic lipogenic and glycolytic gene expression

Primary rat hepatocytes prepared by the collagenase perfusion were plated into Primaria tissue culture dishes in the presence of 1 μM T_3, 1 μM insulin and 10 nM dexamethasone. The cells were maintained in media containing Me_2SO or 100 μM Wy14,643 for 48 hours. Total RNA was prepared from the hepatocytes and analyzed by Northern blot analysis for mRNA coding for S14, FAS and PK. RLS: Rat liver standard.

of Me_2SO, high level of hybridization was observed with probes for S14 mRNA. Addition of 100 μM Wy14,643 to the medium caused a marked decrease in $mRNA_{S14}$ abundance.

The specificity of Wy14,643 on hepatic gene expression was examined with probes for fatty acid synthase (FAS), L-type pyruvate kinase (PK) and β-actin. 100 μM of Wy14,643 exerts strong suppressive effects on both $mRNA_{FAS}$ and $mRNA_{PK}$. On the other hand, mRNA encoding β-actin was not significantly affected. Therefore, Wy14,643 appears to specifically suppress these lipogenic and glycolytic gene expression in primary hepatocyte culture.

The dose response of hepatic $mRNA_{S14}$ and $mRNA_{FAS}$ to Wy14,643 was examined by dot blot hybridization analysis. The quantified results are shown in Figure 10. Wy14,643 suppresses both mRNAs in a dose-dependent manner The approximate ED_{50} for the Wy14,643 effect is < 50 μM.

Effect of Wy14,643 on Transfected S14CAT Activity. The effect of Wy14,643 on S14 gene transcription was evaluated by first transfecting primary hepatocytes with an S14CAT fusion gene plus a thyroid hormone receptor expression vector (MLV-TRβ1) and treating the cells with T_3 (Jump et al., 1993). The S14 gene contains several functional cis-acting elements involved in both the hormonal (T_3, glucocorticoid, retinoic acid and insulin) and nutrient (glucose and PUFA) control of transcription. The location of these various cis-regulatory elements is illustrated in Figure 3.

Following transfection, hepatocytes were treated with T_3 in the absence or presence of 100 μM Wy14,643 for 48 hr. Cells were harvested and assayed for CAT activity. The CAT activity upon Me_2SO and Wy14,643 treatments is correlated with $mRNA_{S14}$ levels from hepatocytes under the same treatments (Figure 11). S14CAT

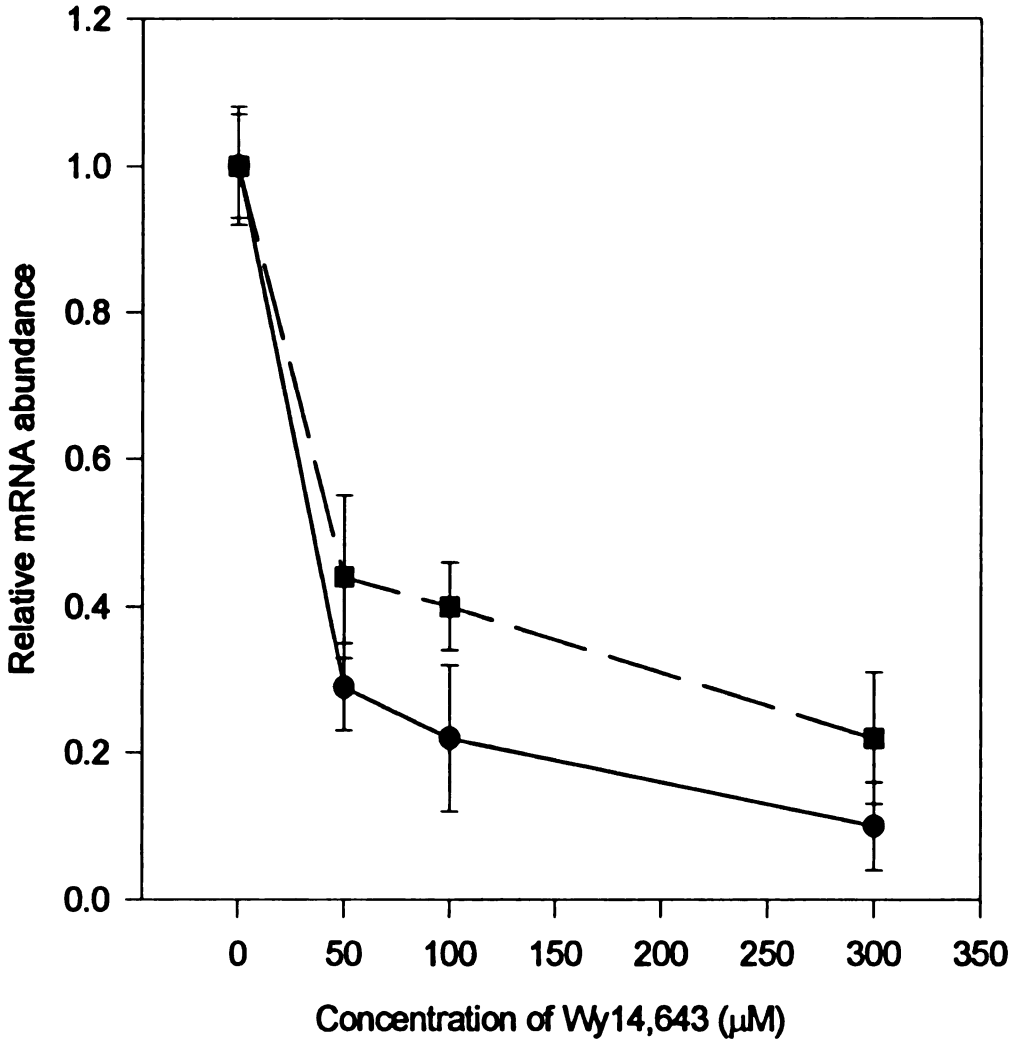


Figure 10. Dose responses of $mRNA_{FAS}$ and $mRNA_{S14}$ to Wy14,643

Primary rat hepatocytes prepared by the collagenase perfusion were plated into Primaria tissue culture dishes in the presence of 1 μM T_3, 1 μM insulin and 10 nM dexamethasone. The cells were maintained in media containing increasing concentrations of Wy14,643 (0 - 300 μM) for 48 hours. Total RNA was prepared from the hepatocytes and analyzed by Northern blot analysis for mRNA coding for S14 (circles) and FAS (squares). The results were quantified by video densitometry and expressed as percentage of DMSO control. Mean ± S. D. N = 3.

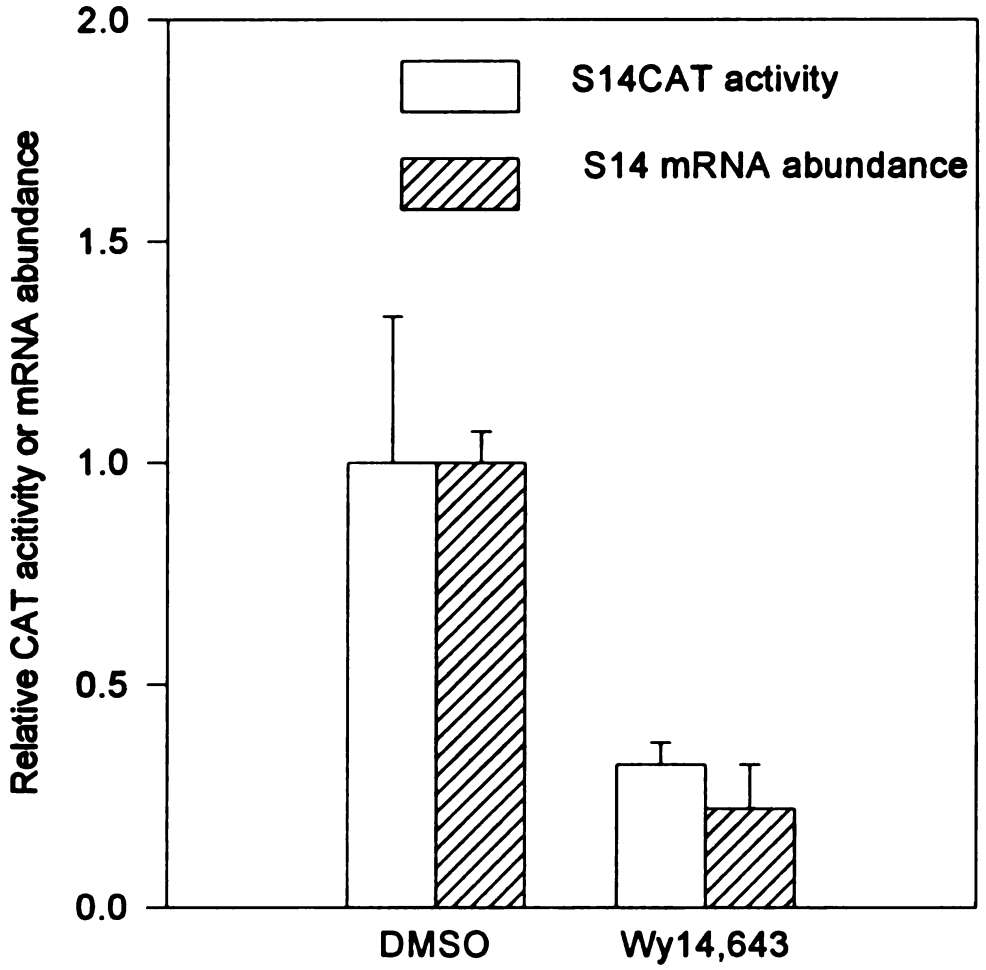


Figure 11. Effect of Wy14,643 on S14 promoter activity

Primary rat hepatocytes were transfected with a T_3 receptor expression vector (MLV-TRb1) and S14CAT124 reporter gene which contains S14 genomic elements extending from -4315 to +19 bp. After transfection, cells were treated with T_3 and WY14,643 (100 mM) as before. After 48 hrs. cells were harvested and assayed for CAT activity. RNA was extracted from cells that were not transfected and $mRNA_{S14}$ measured as described in Figure 9. Results are expressed as percentage of the DMSO control, mean ± SE, N≥4.

activity was suppressed by ≥ 75% as a result of Wy14,643 treatment and paralleled the decline in $mRNA_{S14}$. Thus, Wy14,643 suppressed hepatic $mRNA_{S14}$ by inhibiting S14 gene transcription. This study also indicates that Wy14,643 cis-regulatory elements were located between -4315 and +19 bp relative to the 5' end of the S14 gene. In the absence of cotransfected TRβ1, S14CAT124 exhibits only weak CAT activity, though reproducibly above the background. And the CAT activity is further reduced by addition of Wy14,643 (data not shown).

Effect of mPPARα on S14 gene promoter activity. To determine whether the inhibitory effect of Wy14,643 on S14 gene transcription was mediated by peroxisome proliferator activated receptor (PPAR) (Issemann and Green, 1990), expression vector containing the full length of mouse PPARα was used in transfection studies. Hepatocytes were transfected with S14CAT124, MLV-TRβ1, and increasing amount of mPPARα in the absence or presence of 100 μM Wy14,643. Figure 12 shows that cotransfection of mPPARα inhibits S14CAT124 activity in a dose-dependent manner. The ED_{50} of this inhibition is < 0.7 μg/plate. Substitution of an equivalent amount of the empty vector, i.e., pSG5, or another nuclear receptor, pSG5-RXRα, for pSG5-PPARα was not inhibitory to S14CAT activity (data not shown). At each dose of transfected mPPARα, the addition of Wy14,643 to the medium further amplified the inhibitory effect. In the presence of 100 μM Wy14,643, the ED_{50} of mPPARα is < 0.1 μg/plate. Taken together with the dose response curve of TKCAT223 (Figure 8), 0.2 μg/plate PPAR was used in most of the following transfection experiments because: 1) it was within the linear portion of the dose response curves of both S14CAT and TKCAT constructs; 2) it provided enough signal

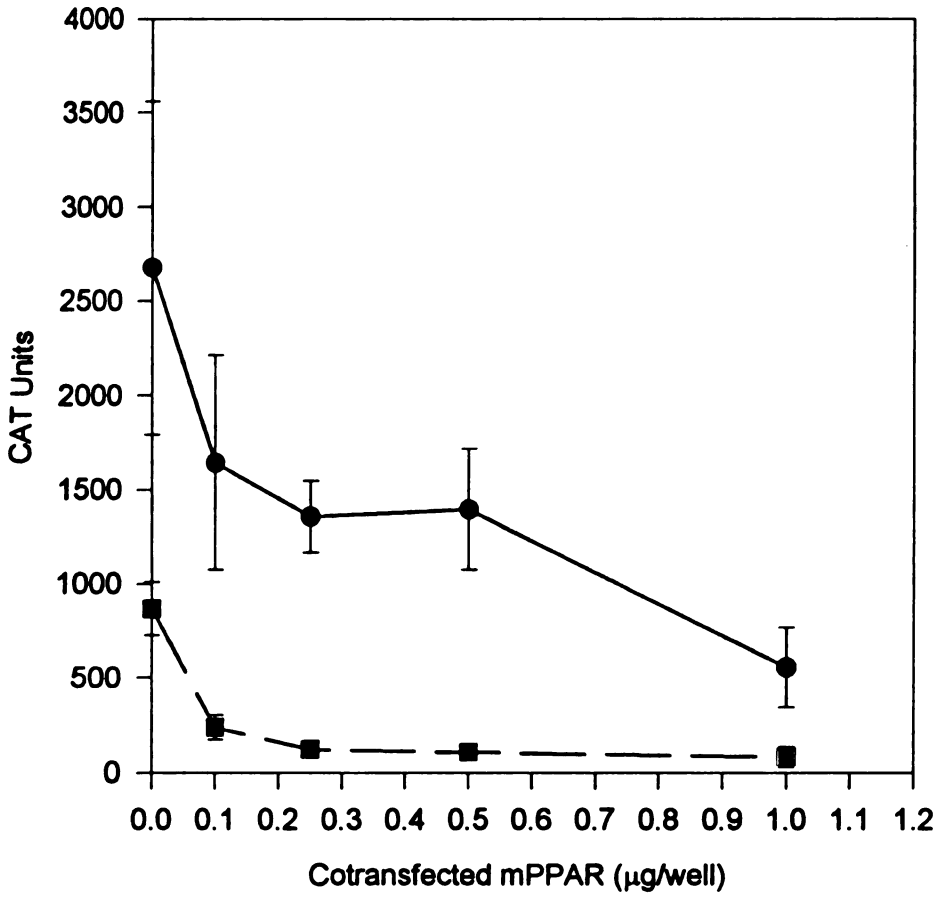


Figure 12. Dose response of S14CAT124 to cotransfected PPAR

Hepatocytes were cotransfected with S14CAT124 (2 µg), MLV-TRβ1 (1 µg) and increasing amount of pSG5-PPAR (0 - 1.0 µg). Cells were treated with 1 µM T_3 and either DMSO (circles) or 100 µM Wy14,643 (squares). After 48 hours, cells were harvested for CAT activity (Mean ± SE, N ≥ 6).

and was easily detectable; and 3) it minimized the quantity of PPAR expression plasmid used in the studies.

Wy14,643 Inhibition Is not Due to Generalized Toxic Effects of Wy14,643 to Hepatocytes. To verify whether the Wy14,643 inhibition of gene expression was only a secondary phenomenon, i.e. caused by any generalized toxic effect to hepatocytes, LDH activity assay was performed to examine the viability of Wy14,643 treated hepatocytes. As shown in Figure 13, Me_2SO causes a slight increase of LDH released to the media by hepatocytes comparing with the cells that are cultured in Me_2SO-free media. However, the level of LDH released to the media by Wy14,643 treated hepatocytes was similar to that released by vehicle treated cells. Moreover, the lack of any significant effect of Wy14,643 on the levels of $mRNA_{\beta\text{-actin}}$ (Figure 9) and the activity of cotransfected RSVCAT (Figure 7) suggests cytotoxicity cannot account for the specific effects of Wy14,643 on the expression of genes encoding AOX, S14, FAS and PK.

Locating the Negative-PPRE (nPPRE) within the S14 5'-Flanking region. In order to determine whether the PUFA and WY14,643 cis-regulatory elements were located in the same region, hepatocytes were transfected with a truncated version of S14CAT fusion gene. S14CAT149 contains only the S14 TRR extending from -2900 to -2500 fused upstream from the S14 proximal promoter extending from -290 to +19. The results are shown in Figure 14. Wy14,643 inhibited the CAT activity by 60%. Cotransfection of PPAR in the absence of Wy14,643 inhibited the CAT activity by 40%. When Wy14,643 and PPAR cotransfection were combined, a strong inhibition (95%) was observed. Therefore, the response pattern of S14CAT149 to Wy14,643, PPAR and the combination of both was almost identical to that seen with S14CAT124. These results

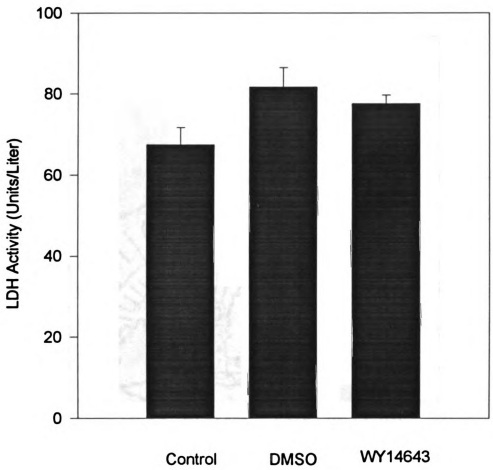


Figure 13. Toxicity assay of Wy14,643 treated hepatocytes

Hepatocytes were treated with vehicle (William's E medium only), DMSO or 100 μM Wy14,643. At the completion of the 48 hour treatment, media were harvested and assayed for lactate dehydrogenase activity as described in Materials and Methods. Results are represented as mean ± SE, N = 3.

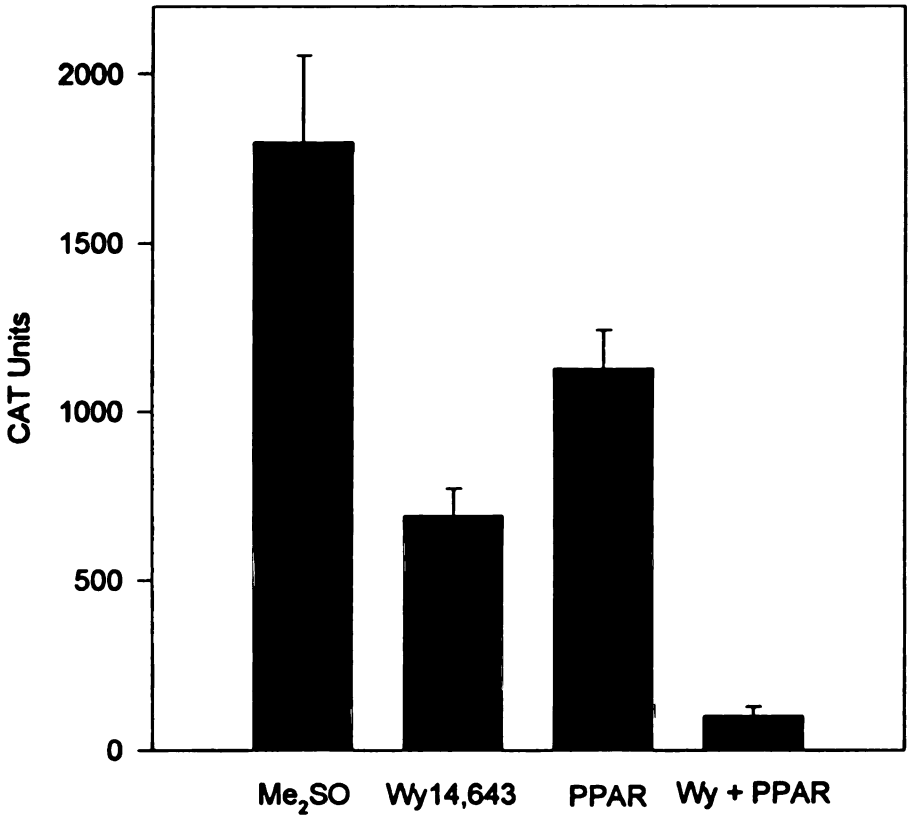


Figure 14. Effects of Wy14,643 and PPAR on S14CAT149

Hepatocytes were cotransfected with S14CAT149 (2 μg) and MLV-TRβ1 (1 μg). Half of the cells were cotransfected with 0.2 μg pSG5-PPAR. Cells were treated with 1 μM T_3 and either DMSO or 100 μM Wy14,643. After 48 hours, cells were harvested for CAT activity(Mean ± SE, N ≥ 6).

indicate that the WY14,643 cis-regulatory elements are located either within the S14 proximal promoter element (-290 to +19), or the S14 TRR (-2900 to -2500), or both regions.

Electrophoretic Gel Shift Analysis of PPAR and AOX-PPRE Interaction. PUFA control of S14 gene transcription appears to involve only the S14 proximal promoter elements between -220 and -80 bp (Jump et al., 1993). Based on the preliminary transfection studies this same region may be involved in peroxisomal proliferator control of S14 gene transcription. Since WY14,643 induction of peroxisomal genes is mediated, at least in part, by activation of PPAR (Issemann and Green, 1990; Green, 1992; Kliewer et al., 1992), an obvious question was if PPAR interacted with PUFA-RE within the S14 proximal promoter. Using labeled AOX-PPRE and nuclear receptors synthesized by an *in vitro* transcription-translation system, gel-mobility shift analysis was performed (Figure 15). Neither PPARα (lane 1) nor RXRα (lane 2) alone showed binding on AOX-PPRE (lanes 1 and 2). However, the combination of PPAR and RXR (lane 3) leads to the formation of a heterodimeric complex consisting of PPAR-RXRα (Keller et al., 1992; Kliewer et al., 1992; Gearing et al., 1993). Addition of a 100-fold molar excess of unlabeled AOX-PPRE effectively competed for the formation of the PPAR-RXR complex (lane 4). Interestingly, addition of DNA elements from the S14 promoter extending from -120 to -80 (Y-box, lane 5), -220 to -120 (HNFB2, lane 6) or from -290 to +19 (lane 7) failed to compete for binding. Moreover, attempts to bind PPAR/RXR directly to the S14 proximal promoter elements were unsuccessful (data not shown). Based on these observations, PPAR does not bind directly to the S14 proximal promoter.

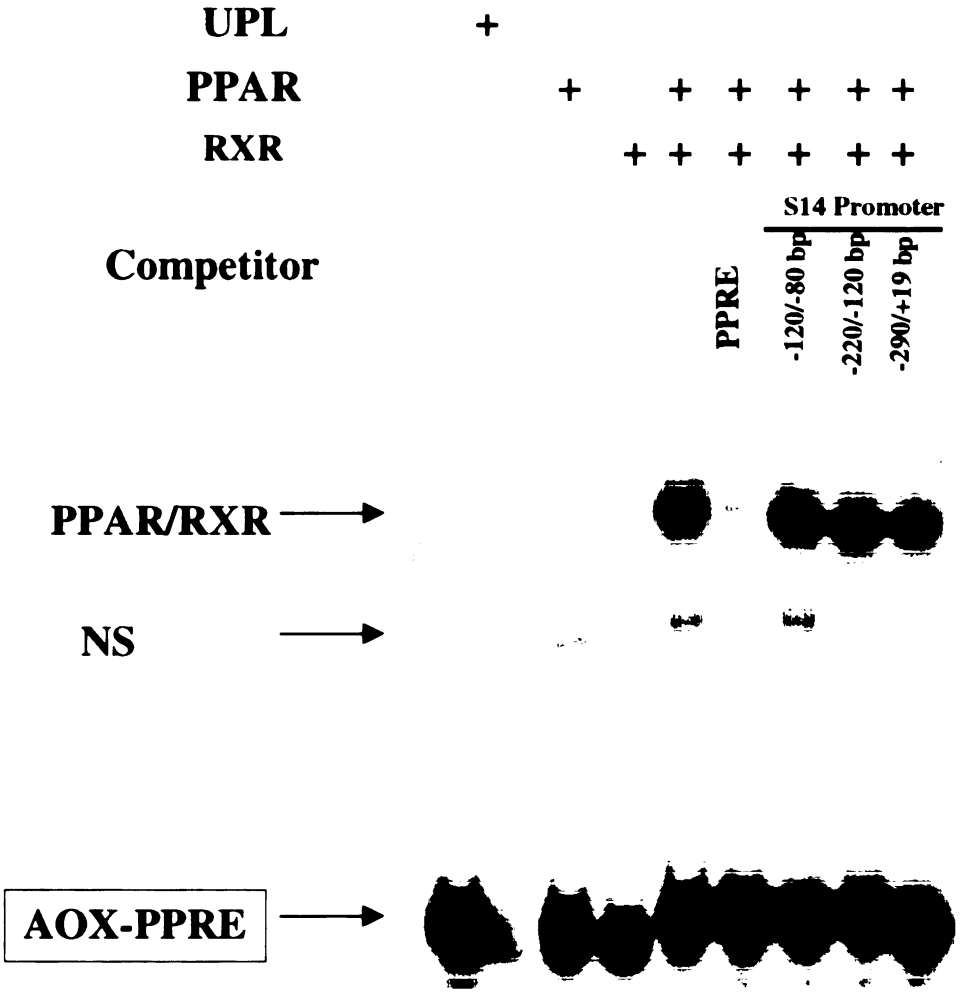


Figure 15. PPAR does not bind S14 proximal promoter

RXRα and mPPARα were synthesized *in vitro* by programming theTNT cell lysate with plasmids containing the full length RXRα and mPPAR. The AOX-PPRE was end labeled with ^{32}P by T_4 poly-nucleotide kinase. Gel shift analysis was carried out as described in Materials and Methods. The competition assays using competitor DNA sequences were at 100-fold molar excess. NS: Non-specific binding.

Discussion

I have examined the role played by a potent peroxisome proliferator, Wy14,643, and PPARα in the control of gene expression in primary hepatocytes. The peroxisome proliferative effects of the potent peroxisome proliferator, Wy14,643, has been reported in a number of cell systems, such as CHO (Gottlicher et al., 1992), COS (Issemann and Green, 1990; Schimdt et al., 1992), Hela (Dreyer et al., 1992) and Hepa 1 cells (Sher et al., 1993). In this study, I have shown Wy14,643 is able to induce an mRNA encoding a peroxisomal enzyme, i.e. AOX, in cultured primary hepatocytes in a dose-dependent manner, marked by the elevated level of $mRNA_{AOX}$ (Figures 6). The stimulatory effect of Wy14,643 on AOX gene expression was mediated by the interaction between PPARα and AOX-PPRE, as demonstrated by transfection experiments (Figures 7).

My thesis project was designed to examine the involvement of PPAR in PUFA regulation of hepatic S14 gene transcription (Jump et al., 1993). PPAR has been implicated as the nuclear transcription factor mediating both Wy14,643 and fatty acid control of gene expression (Gottlicher et al., 1992; Keller et al., 1992; Jump et al., 1993). Having determined the effectiveness of Wy14,643 and PPAR in inducing peroxisomal gene expression in cultured hepatocytes, I examined the involvement of these factors in the regulation of hepatic lipogenic and glycolytic gene expression. Hepatocyte S14, FAS and PK gene expression was inhibited by Wy14,643 within a dose range used by others to demonstrate the induction of such peroxisomal enzymes as acyl-CoA oxidase (Issemann and Green, 1990; Tugwood et al., 1993), CYP4A6 gene (Muerhoff et al., 1992) and a peroxisomal hydratase-dehydrogenase gene (Zhang et al., 1993) (Figure 9), and the extent and pattern of inhibition was comparable to that caused by PUFA (Figure 10, Jump et al.,

1993). In transfection studies, Wy14,643 inhibited the S14CAT124 activity (Figure 11). Because the effect of Wy14,643 on S14CAT activity paralleled the decline in $mRNA_{S14}$, it strongly suggests that the principal mechanism of Wy14,643 action was at the level of S14 gene transcription, although the possibility of regulation at other levels of gene expression can not be completely ruled out based on current experimental results. These stimulatory or inhibitory effects were gene-specific because the level of $mRNA_{\beta\text{-actin}}$ (Figure 9) and the activity of cotransfected RSVCAT101 (Figure 7) was not affected by Wy14,643. Toxicity analysis further confirmed that the slight toxicity caused by Wy14,643 treatment was not sufficient to account for the induction of AOX or inhibition on S14, FAS and PK genes (Figure 13).

The similarities in the effects of PUFA and Wy14,643 on the levels of mRNAs encoding AOX, S14, FAS and PK imply that these two classes of compounds may exert their effects on gene expression via the same molecular mediator, i.e. PPAR. A truncated S14CAT construct S14CAT149, which contains the -2900 to -2500 bp S14 TRR and the proximal 290 bp of S14 promoter, exhibited a pattern of response to Wy14,643 and PPAR that was almost identical to that of S14CAT124, which contains the full length of the first 4 kb 5'-flanking region upstream of the S14 promoter (Figure 14). It appeared that the PPAR *cis*-regulatory element(s) is located either within the S14TRR, or the S14 proximal promoter, or both. If PPAR was involved in the PUFA control of S14 gene transcription, then PPAR might bind to S14 PUFA-REs within the S14 proximal promoter and affect gene transcription. Surprisingly, PPAR either alone or in combination with RXR did not bind S14 promoter elements (Figure 15). This finding suggests that if PPAR is involved in

mediating PUFA effects on S14 gene transcription it does not require direct binding to S14 promoter elements.

Finding that PPAR does not bind S14 promoter elements may indicate that: 1) The PPAR-mediated effects of Wy14,643, and possibly PUFA, on S14 gene do not require a direct PPAR-DNA interaction; 2) other PPAR isoforms might be operative; or 3) PPAR might target S14TRR, and not the PUFA-RE within the S14 proximal promoter. First, it has been well documented that nuclear receptor action on gene transcription does not always require receptor binding to DNA (Yen-Yang et al., 1990; Husmann et al., 1991). PPAR might sequester key transcription factors that are required for S14 promoter function. In the second case, several PPAR isoforms have been cloned from human, rodents and Xenopus (Issemann and Green, 1990; Gottlicher et al., 1992; Keller et al., 1992; Schmidt et al., 1992; Zhu et al., 1993). In this study, I have only examined an α isoform from mouse. Whether other PPAR isoforms interact with the S14 promoter remains to be determined. Finally, the PUFA inhibition of S14 gene transcription is independent of the S14TRR (Jump et al., 1993). Preliminary evidence does not exclude the possibility that Wy14,643 and PPAR target the S14TRR region (Figure 14). Before the extent of PPAR involvement can be determined in PUFA control of S14 gene transcription, the precise site(s) of PUFA-RE and PPRE controlling S14 gene transcription must be determined and the trans-acting factors binding these elements have to be identified.

Chapter 3. The Molecular Basis for Wy14,643/PPAR Inhibition of S14 Gene Transcription in Hepatocytes

Introduction

Previous studies have shown that a potent peroxisome proliferator, Wy14,643, suppresses both $mRNA_{S14}$ level and S14CAT activity in cultured primary rat hepatocytes, via activation of PPARα. The similar extent of inhibition of $mRNA_{S14}$ and S14CAT activity suggests the control is at the gene transcriptional level. Wy14,643 also specifically inhibits the expression of FAS and PK gene expression. Because of the similar responses of these genes to Wy14,643 and to PUFA, plus the observations by other researchers, including: 1) feeding rats high fat diets induces peroxisomal enzymes (Neat et al., 1980; Thomassen et al., 1982; Flatmark et al., 1988; Rodriquez et al., 1994); and 2) *in vitro* transfection studies show that fatty acids activate PPARα (Gottlicher et al., 1992; Schmidt et al., 1992; Kaikaus et al., 1993; Keller et al., 1993; Kliewer et al., 1994; Amri et al., 1995; Yu et al., 1995; Schoonjans et al., 1995), it was reasonable to envision that PPARα might be involved in, and could be the molecular mediator of PUFA regulation of hepatic gene transcription. In the studies reported in this chapter, I focused on defining the target of peroxisome proliferator regulation of S14 gene transcription. In contrast to our expectation, the cis-regulatory targets for Wy14,643 and PPARα action were mapped to the S14TRR and not to the proximal promoter region containing the negative PUFA-response elements (nPUFA-RE) (Jump et al., 1993). Based on these findings, PPARα does not appear to be the mediator of PUFA regulation of hepatic S14 gene expression.

Furthermore, by transfection and gel-mobility shift analysis, I looked into the molecular mechanism of the Wy14,643/PPAR inhibition of S14 gene transcription.

Materials and Methods

Plasmids: The construction of all S14CAT reporter genes and most other plasmids have been mentioned previously (Jump et al., 1993; Chapter 2). These plasmids include reporter plasmid TKCAT223 and expression plasmids pMLVTRβ1, pSG5-PPARα and pSG5-RXRα. A plasmid that has not been used in the previous chapter is reporter TKCAT222, which contains the S14 TRR (-2.9/-2.5 kb), fused upstream of the TK minimal promoter (Jump et al., 1993).

Hepatocyte Culture and Transfections: Primary rat hepatocytes were prepared and transfected as previously mentioned (Chapter 2). After transfection and different treatments, cells were harvested for protein assay and CAT activity assay. CAT activity: CAT Units = CPM of ^{14}C- butylated chloramphenicol/hour/100 mg protein.

Gel Mobility Shift Assays: The gel mobility shift assay was performed as described previously (Chapter 2).

RESULTS

Wy14,643/PPARα and PUFA Target Different S14 Gene Regulatory Elements. My previous studies have shown that the cis-regulatory elements targeted by PPARα and Wy14,643 are located in the TRR (-2900 to -2500 bp) or the proximal 290 bp region of S14 gene. To precisely define the cis-regulatory elements, progressive promoter deletion analysis was performed. The same strategy was used to localize the nPUFA-RE within the S14 proximal promoter region (Jump et al., 1993). A series of

promoter deletions was prepared from the 290 bp 5'-flanking sequence upstream of the S14 transcription start site. The 3' ends of these elements all point at + 19 bp from the transcription start site. The 5' ends were progressively truncated at -220, -120, -80, and -40 bp, respectively. In each construct the S14TRR region was retained to ensure high transcriptional activity and to allow for an examination of any inhibitory effects of Wy14,643 and PPARα on S14CAT activity. Hepatocytes were transfected with these plasmids with or without cotransfected mPPARα expression vector. The cells were treated with either Me_2SO or Wy14,643. The CAT activities of different treated hepatocytes were assayed and shown in Figure 16A. As seen in the previous transfection experiments, both Wy14,643 and PPARα inhibited S14CAT149 by ~ 50% and the combination inhibited S14CAT149 activity by 85%. This same pattern of control was seen with all S14 deletion constructs used in the study. The percentage of inhibition was summarized and compared with that by PUFA (Jump et al., 1993) in Figure 16B. S14 proximal promoter elements when shortened to -120 and -80 bp lost responsiveness to PUFA while still appeared to be sensitive to Wy14,643 and PPARα. The results of this study suggested that the PUFA-RE (at -220/-80 bp) was not involved in Wy14,643/ PPARα-mediated control of S14 gene transcription.

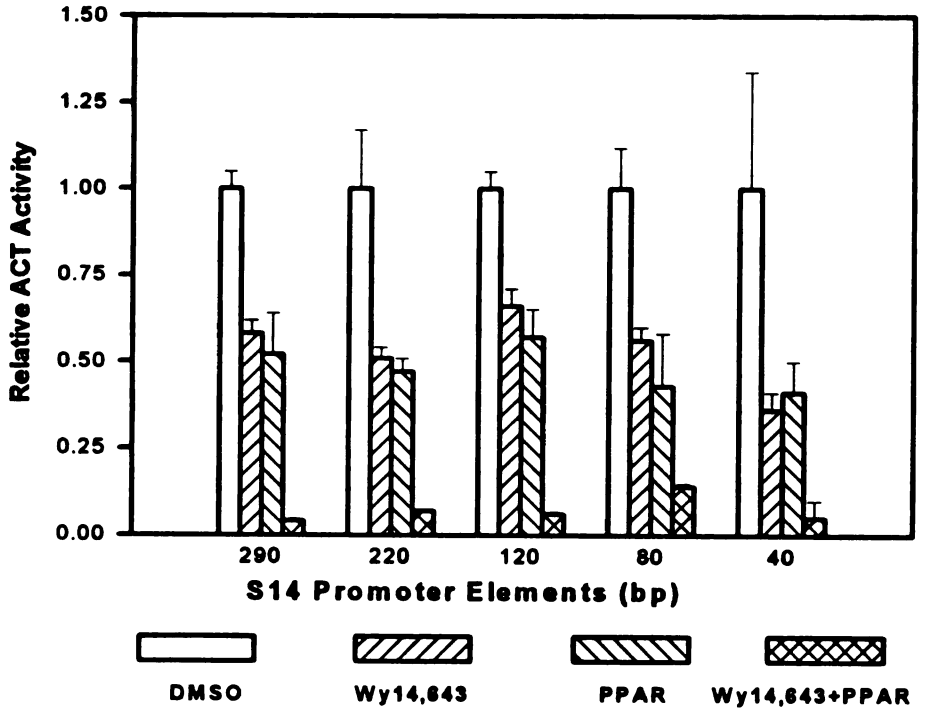
Locating the Wy14,643/PPAR Target of S14 Gene. The minimal elements required for the S14 gene to be responsive to Wy14,643 and PPARα were the elements within the proximal promoter region (-40/+19 bp) containing only a TATA box and the upstream TRR. Consider the omnipresence of TATA box and the specificity of PPAR control of gene transcription, it would be hard to speculate that the target for gene-specific inhibitory effect of PPAR on S14 gene is this 40 bp sequence. Therefore the S14

Figure 16. S14 promoter deletion analysis

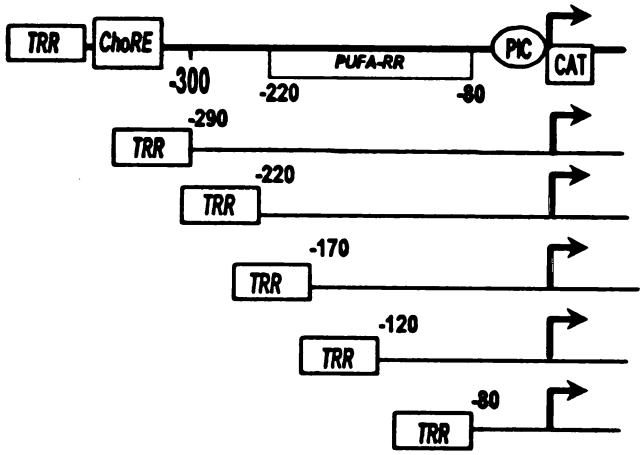
A. Primary hepatocytes were cotransfected with various promoter constructs and MLVTRβ1 as described before. These cells were treated with T_3 and DMSO or T_3 and 100 μM-Wy14,643. Half of the hepatocytes also received pSG5-PPARα (0.2 μg/culture) and were treated with either DMSO or Wy14,643. T_3-stimulated CAT activity for control (DMSO) treated cells transfected with S14CAT124, 149, 155, 156 or 158 was 2293 ± 220, 1799 ± 255, 547 ± 17, 633 ± 76 and 69 ± 8.2 CAT Units, respectively. Results are expressed as Relative CAT Activity (Mean ± SE, $N \geq 6$). An ANOVA test for statistical significance of the effect of Wy14,643, PPARα or the combination of these treatments, all were $p<0.001$.

B. Schematic representation of the 5' regulatory region controlling the S14 gene transcription. TRR, thyroid hormone response region (at -2.8 to -2.5 kb); ChoRE, carbohydrate response element (at -1.6 to -1.4 kb); PIC, preinitiation complex binding at the TATA-box; PUFA-RR, PUFA-response region (at -220/-80 bp). The deletion constructs are shown numbered, 124, 149, 155, 156 and 158. Each construct contains the same S14TRR upstream from the S14 promoter element. The 3' end point (at +19 bp) is common to all constructs, the 5' end points vary (at -290, -220, -120 and -80 bp, respectively).

A.



B.



ID#	% Inhibition PUFA	Wy
124	66	58
149	40	42
155	40	49
156	24	43
158	8	44
159	0	65

Figure 16

TRR was tested as the prospective target of Wy14,643/ PPARα control by fusing this element (-2.9/-2.5 kb) to the heterologous thymidine kinase promoter (TKCAT222). The specificity of Wy14,643/ PPARα effects on transcription was examined by comparing the CAT activity of TKCAT222 with that of: 1) an enhancer-less TKCAT fusion gene (TKCAT202); and 2) a S14CAT fusion gene containing the S14TRR fused upstream from the -290/+19 bp region of the S14 promoter (S14CAT149). The results are shown in Figure 17. TKCAT202 was not significantly affected by Wy14,643 or cotransfected PPARα (Figure 17 inset). Inserting the S14TRR upstream from the TK promoter conferred high levels of T_3-induction (>50-fold) of CAT activity (Jump et al., 1993). Both Wy14,643 and PPARα inhibited TKCAT222 by ~50%, and the combination of these treatments further inhibited TKCAT222 activity by more than 90%. This pattern of control is identical to that seen with S14CAT149. The results of this study suggests that the S14TRR is the target of Wy14,643/ PPARα action. It appears that the direction of control and sensitivity to Wy14,643 and PPARα regulation is enhancer-dependent. While the S14TRR confers negative control, the AOX-PPRE confers positive control to the TKCAT fusion gene following Wy14,643/ PPARα treatment. These studies confirm and extend the deletion studies (Figure 16) by showing that the S14TRR is sufficient and necessary for the negative effect of Wy14,643/ PPARα on the S14 or TK promoter activity. Therefore the cis-regulatory elements for Wy14,643/PPARα and PUFA control of S14 gene transcription are functionally and spatially distinct.

mPPARα Does Not Bind the S14TRR Directly. To determine if PPARα-mediated effects on S14 gene transcription were due to direct binding to the S14TRR, gel

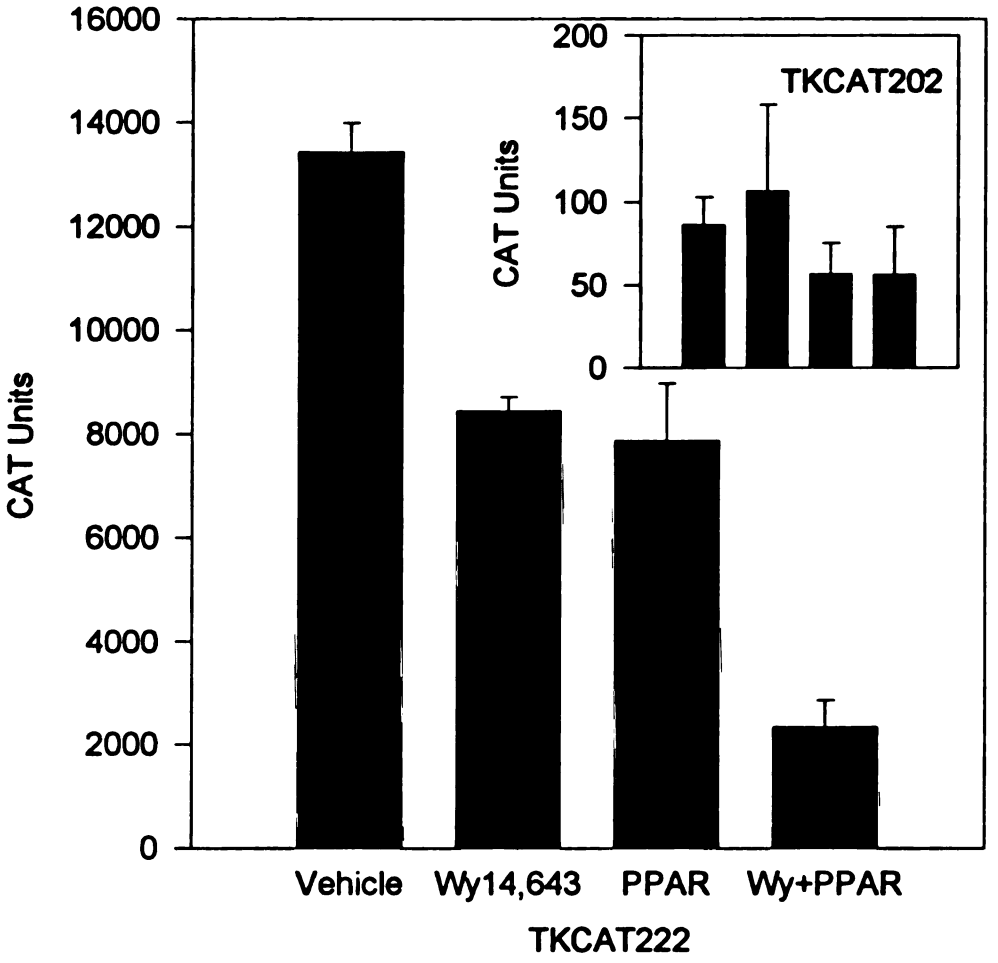


Figure 17. Effects of Wy14,643 and PPAR on TKCAT222

Hepatocytes were cotransfected with TKCAT222 (2 μg) and MLV-TRβ1 (1 μg). Half of the cells were cotransfected with 0.2 μg pSG5-PPAR. Cells were treated with 1 μM T_3 and either DMSO or 100 μM Wy14,643. After 48 hours, cells were harvested for CAT activity(Mean ± SE, N ≥ 6). Inset: TKCAT202 was transfected to hepatocytes with the same procedure.

mobility shift analysis was performed using ^{32}P-labeled AOX-PPRE as a probe (Figure 18). Neither PPARα or RXRa alone bind the AOX-PPRE. The combination of these receptors bind to AOX-PPRE as a heterodimer. This observation is consistent with previous reports (Kliewer et al., 1992; Tugwood et al., 1992; Gearing et al., 1992; Jump et al., 1995). Addition of a 100- molar excess of unlabeled AOX-PPRE effectively competes for the formation of the PPARα/RXR complex. In contrast, a 100-fold molar excess of the S14TRR failed to compete for binding. No competition was seen with even a 500-fold molar excess of TRR (not shown). The S14TRR region contains 3 TREs, which consist of direct repeats of AGGTCA-related motifs separated by 4 nucleotides (DR-4) (Liu and Towle, 1994). These elements, also known as far upstream regulatory elements (FUR 10, 11 and 12) did not compete for PPARα/RXRα binding (data not shown). PPARα/RXRα also did not bind directly to a canonical DR-4 (gatcctcAGGTCAcaggAGGTCAgag, see Figure 20). These studies show that PPARα either alone or with RXRα does not bind the S14 FUR elements or other DNA elements within the -2.9 to -2.5 kb S14TRR.

PPARα Suppresses Hepatic S14 Gene Expression by Functionally Interfering with TR/RXR Action. Since PPARα did not interact directly with the TRR, we speculated that PPARα might affect T_3 action indirectly. It has been reported that co-transfected PPAR effects on T_3-dependent gene transcription are eliminated by elevating cellular RXR levels (Juge-Aubry et al., 1995). To determine if PPARα action on S14CAT activity was affected by hepatocellular levels of other receptors, increasing amount of TRβ1 (as MLVTRβ1) or RXRα (as pSG5-RXRα) was cotransfected with a constant

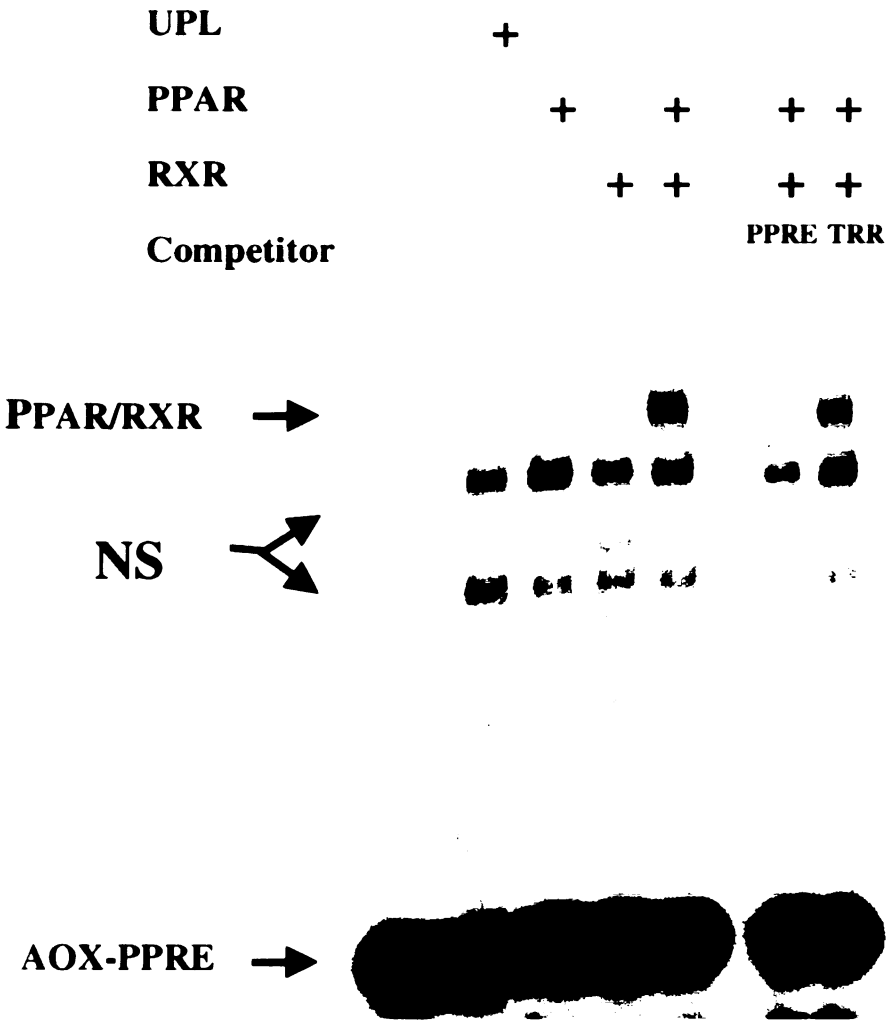


Figure 18. PPAR does not bind The S14 TRR

Gel shift analysis was performed using ^{32}P-labeled acyl-CoA oxidase (AOX-PPRE) oligonucleotide and *in vitro* transcribed/translated mPPARα and RXRα as described in Materials and Methods. Competition assays were performed in the presence of a 100-fold molar excess of unlabeled AOX-PPRE or S14TRR. The results are representative of 4 separate studies. NS: Non-specific binding.

amount of pSG5-PPARα (0.5 μg/plate) and TKCAT222 (1 μg/plate). The results are shown in Figure 19. While cotransfected TRβ1 is required for T_3 control of transfected TKCAT222, increasing hepatocellular levels above the 1 μg/plate level did not enhance T_3 activation or affect PPARα-mediated inhibition of TKCAT222 activity. Cotransfected RXRα is not required for T_3-mediated control of TKCAT222 activity in hepatocytes cotransfected with MLV-TRβ1 (Jump et al., 1993, 1995). However, increasing hepatocellular RXRα levels by cotransfecting pSG5-RXRα (at 1 μg/plate) was sufficient to override the inhibitory effect of PPARα on TKCAT222 activity. This pattern of control suggests that RXR might be limiting in primary hepatocytes.

To determine how this interference might occur, gel shift analysis was used to examine the effect of PPARα on TRβ binding to a DR-4 (Figure 20). While RXRα fails to bind a DR-4, TRβ binds as a monomer. Addition of both TRβ and RXRα yields a heterodimer binding on the DNA element and caused a decrease of the amount of TR homodimer. When increasing amount of PPARα was added together with TRβ and RXRα, the shifted band representing TR/RXR heterodimer diminished and the intensity of lower band, which represented TR homodimer, increased. Thus, addition of PPARα inhibited heterodimer formation and favored TR monomer formation. Addition of unprogrammed reticulolysate lysate had no effect on binding of TRβ as monomers or TRβ/RXRα heterodimers. Thus, PPARα interferes with TR/RXR binding to DR-4. This observation essentially confirms and extends the report by Juge-Aubry, et al (1995) by showing that PPARα inhibits TRβ/RXRα binding to a DR-4.

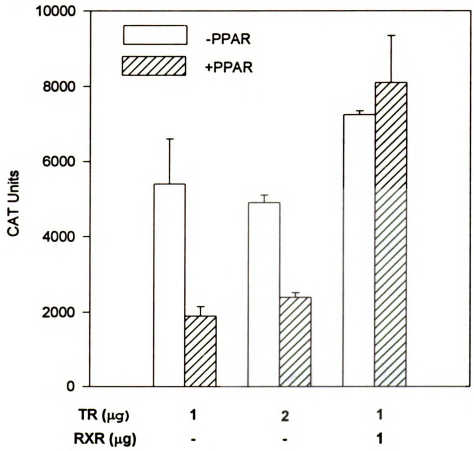


Figure 19. Cotransfection of RXR eliminates PPAR inhibition of S14TRR activity

Hepatocytes were cotransfected with TKCAT222 (2 mg) and MLV-TRβ (ranging from 1 to 2 μg/well). Cells were also cotransfected without [open bars] or with pSG5-PPARα (0.5 μg/well) [solid bars]. All cells received T_3 to induce TKCAT222 activity. The results were pooled from 3 separate experiments and are expressed as CAT Activity, Units, (Mean ± SE, n=9).

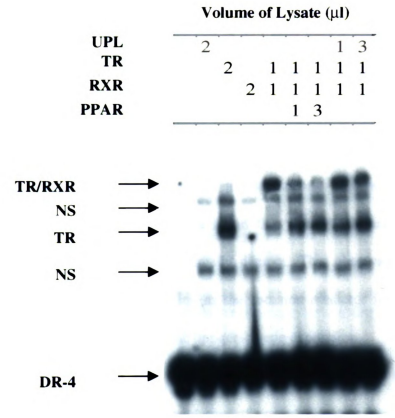


Figure 20. PPAR interrupts TR/RXR heterodimerization

Gel shift analysis was used to examine the effect of PPARα on TR/RXR binding as described in Materials and Methods. A DR-4 (GATCCTCAGGTCACAGGAGGTCAGAG) was end labeled and used for gel shift analysis. The Figure indicates the volume of unprogrammed reticulolysate and in vitro translated TRβ, RXRα and PPARα added per assay. The location of labeled DR-4, T_3 receptor monomers (TR) and TR/RXR heterodimers are shown by the arrows. This gel shift is representative of 4 separate studies. NS: Non-specific Binding. UPL: Unprogrammed Cell Lysate.

DISCUSSION

The studies reported in this chapter address two questions: 1) Was PPARα the mediator of PUFA regulation of hepatic lipogenic gene transcription? and 2) What was the molecular basis of Wy14,643 and PPAR inhibition of S14 gene transcription? For PPARα to be a mediator of PUFA action, two criteria must be satisfied: 1) both PPARα and its activators must inhibit S14 gene transcription, and 2) the cis-regulatory targets for PPARα and its activators must map to the PUFA-RE within the S14 promoter (Jump et al., 1993). Mapping the nPPRE to the nPUFA-RE would provide strong evidence for PPARα serving as the mediator of PUFA action. It is found that the nPPRE and nPUFA-RE within the S14 gene are functionally and spatially distinct. This observation makes it difficult to envision how PPARα can function as the common mediator for both PUFA and peroxisome proliferator control of hepatic lipogenic gene expression.

PPARα is the predominant subtype expressed in rodent liver and this subtype accounts for the peroxisome proliferator regulation of several enzymes involved in lipid metabolism (Lee et al., 1995). Fatty acids appear to be activators of PPARα only under conditions of lipid-overload, which occurs following peroxisome proliferator treatment, high fat feeding, diabetes mellitus, starvation or pathophysiological states when hepatic mitochondrial β-oxidation is suppressed, i.e., alcoholic liver disease (Kaikaus et al., 1993; Lee et al., 1995). PUFA mediated suppression of lipogenic gene transcription is rapid, occurs within hours of PUFA administration and precedes changes in acyl CoA oxidase mRNA levels (Jump et al., 1993, 1994; Clarke and Jump, 1994; Liimatta et al., 1994). Other arguments against PPARα as the common mediator for PUFA and peroxisome

proliferator action include the finding that PPARs are activated by monounsaturated and polyunsaturated fatty acids and to a lesser extent by saturated fatty acids (Gottlicher et al., 1992, 1993; Keller et al., 1993). Lipogenic enzyme gene expression is suppressed by PUFA, but not affected by saturated or monounsaturated fatty acids (Clarke et al., 1990; Jump et al., 1993, 1994; Clarke and Jump, 1994; Liimatta et al., 1994). Certain peroxisome proliferators, like nafenopin, benzafibrate and MEDICA 16, actually stimulate lipogenic as well as peroxisomal enzyme gene expression (Hertz et al., 1991). Taken together, these studies suggest that fatty acids might regulate two pathways. One involves PPARα and may function in states of lipid overload. The other pathway involves ill-defined PUFA-regulatory factors (PUFA-RF) that are activated by PUFA ingestion. In contrast to PPARα, PUFA-RF do not target the S14TRR (Jump et al., 1993).

The second part of this study focused on defining the molecular basis of PPARα inhibition of S14 gene transcription. The transfection and gel shift studies show that PPARα and activators of PPARα, like Wy14,643, target the S14 TRR. The mechanism of inhibition is explained in Figure 21. It appears that the PPAR inhibition of S14 gene transcription is due to an interference of PPARα with TRβ/RXRα function at the TREs. Thyroid hormone induced S14 gene transcription requires recruitment of RXR, which is limiting in the cell. The gel mobility shift studies indicated that PPARα inhibited TRβ/RXRα binding. Cotransfected RXRα reversed the PPARα-mediated inhibition of T_3 activation of S14 gene transcription (Figures 19 and 20).

The interaction of PPARα with other transcription factors has been reported previously (Hertz et al., 1991, 1995; Krey et al., 1995; Keller et al., 1995; Bogazzi et al., 1994). For example, PPAR/RXR interact with Sp1 to synergistically induce AOX gene

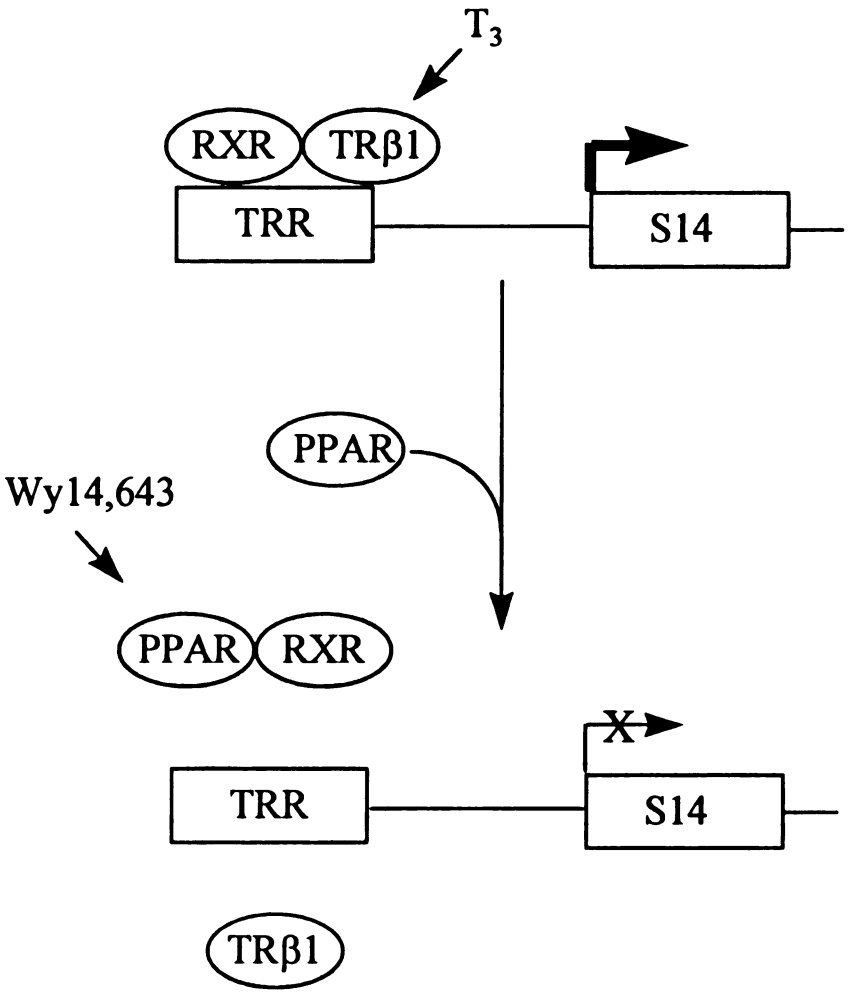


Figure 21. Model of Wy14,643/PPAR inhibition of S14 gene transcription

T_3 is the major inducer of S14 transcription. The action of T_3 requires TR/RXR heterodimer binding to S14 TRR. PPAR competes for the limited amount of RXR in hepatocytes and interrupts the formation of TR/RXR heterodimer, thus blocks the T_3 induction of S14 transcription.

transcription (Krey et al., 1995). In contrast, competitive binding of PPAR/RXR heterodimers to estrogen response elements in the vitellogenin A2 promoter or to the HNF4 site in the apolipoprotein CIII promoter lead to inhibition of transcription (Keller et al., 1995; Hertz et al., 1995). Both these examples require PPAR/RXR to bind PPREs to exert their effect on gene transcription. Other reports indicate that PPAR effects on T_3-regulated gene transcription may not involve direct DNA binding. Bogazzi and coworkers first reported that PPAR cotransfection interfered with T_3 control of malic enzyme and thyroid stimulating hormone b1 gene transcription (1994). Their studies suggested that PPARα heterodimerized with TRβ to form inactive complexes that prevent TR/RXR binding to DNA. In contrast, Juge-Aubry et al., showed that PPARα interfered with T_3 regulated gene transcription by forming heterodimers with RXRa in solution (1995). The interaction between PPAR and RXR was not dependent on PPAR/TR heterodimerization or competition for DNA binding. Under these circumstances, endogenous RXR or TR-auxiliary proteins (TRAP) were limiting. The studies summarized in this chapter show that overexpression of RXRα, but not TRβ1, overrides the negative effect of PPARα on TKCAT reporter genes containing the S14TRR (Figure 19) and that PPARα interfered with the formation of the TR-RXR heterodimer on the S14TRE (Fur 10, 11 and 12) and a canonical DR-4 (Figure 20). These findings are consistent with the report by Juge-Aubry and coworkers and suggest that under the conditions of hepatocyte transfection, RXR is limiting. PPARα inhibits S14 gene transcription by inhibiting TR/RXR heterodimer formation on the S14TREs. The finding that Wy14,643 acting through PPARα affects T_3 receptor action suggests that under conditions of hepatic lipid overload, i.e. starvation and

diabetes, PPARα may play an important role in modulating T_3 regulation of hepatic lipogenic gene expression.

Chapter 4. PUFA and PPAR Regulate Hepatic Gene Transcription via Independent Pathways

Introduction

Two lines of evidence led us to the hypothesis that PPAR might be the molecular mediator of PUFA inhibition of S14 gene transcription: 1) PPAR is activated by fatty acids in transfection studies; 2) PUFA and peroxisome proliferators have similar stimulatory effects on peroxisomal and microsomal fatty acid oxidation pathways. In the previous studies, I focused on defining the molecular mechanism of Wy14,643/PPAR inhibition of hepatic S14 gene transcription. The *cis*-regulatory elements of Wy14,643/PPAR have been localized to the S14 TRR, which is far upstream of the PUFA-RE.

In the studies that are reported in this chapter, the role of PPARα in PUFA-regulation of mRNAs encoding hepatic lipogenic, microsomal and peroxisomal enzymes is examined. I assessed PUFA regulation of the S14 gene and fatty acid synthase, models for lipogenic gene expression, and acyl CoA oxidase (AOX) and cytochrome P450A2 (CYP4A2), enzymes involved in peroxisomal and microsomal fatty acid oxidation, respectively. The recently developed PPARα-knockout mouse (Lee et al., 1995) was used to determine whether PPARα mediates PUFA regulation of hepatic AOX, CYP4A2, S14 and FAS gene expression. This work shows that while PPARα is required for the PUFA-mediated induction of both AOX and CYP4A2 gene expression, it is not required for the PUFA-mediated inhibition of either S14 or FAS gene expression. These and other studies indicate that PUFA regulation of hepatic gene transcription involves at least two distinct pathways, a PPARα-dependent and a PPARα-independent pathway.

Materials and Methods

Animals and Diets. Male Sprague-Dawley rats (125-150 g) were obtained from Charles River Breeding Laboratories (Kalamazoo, MI). Male C57BL/6N X Sv/129 mice (25-35g), F_6 homozygote wild-type (+/+) or knockout (-/-) were used for one of the feeding studies (Lee et al., 1995). Rats and mice were maintained on Teklad chow diet. In all feeding studies, rats and mice were meal-trained to a high carbohydrate diet as previously described (Jump et al., 1993, 1994). The test diets consisted of a high carbohydrate (58% glucose) rat meal (ICN, Cleveland, OH) supplemented with either 10% (wt/wt) of complex fats [triolein, olive, fish (menhaden) oil], fatty acid ethyl esters [eicosapentaenoic acid or docosahexaenoic acid (Southeast Fisheries Science Center, Charleston, SC)] or 0.2% gemfibrozil (Sigma, St. Louis, MO). All diets were supplemented with 0.1% butylated hydroxytoluene to prevent oxidation of fats (Jump et al., 1994). The composition of the fats used in the feeding studies is illustrated in Table 1 (Jump et al., 1994).

Plasmid Construction and Primary Hepatocytes. The construction of the reporter gene with the rat acyl-CoA oxidase (AOX) PPRE fused upstream from the thymidine kinase promoter (TKCAT223) was described previously (Chapter 2, Jump et al., 1993, 1995; Ren et al., 1996).

Primary hepatocytes were obtained from rat liver by the collagenase perfusion method and transfected with specific DNAs in the presence of Lipofectin. Hepatocytes were treated with triiodothyronine (T_3) along with specific fatty acids or peroxisome proliferators [WY14,643 or gemfibrozil dissolved in Me_2SO] as mentioned previously (Chapter 2). After 48 hours of treatment, hepatocytes were analyzed for protein and CAT

Table 1. Percentage composition of fatty acid in dietary fats

Fatty acids	Olive oil	Menhaden oil	C20:5 ester	C22:6 ester
Saturated	13.5	26.0		
Monounsaturated	73.7	21.4		
PUFA	8.4	43.4		
16:0	11.0	15.9		
18:0	0.2	2.9		
18:1	72.5	7.4		
18:2	7.9	1.1		
18:3	0.6	0.7		
20:4		0.8		
20:5		16.1	90.5	1.1
22:6		11.2	0.2	85.7

activity. CAT activity is defined as CAT units = counts/min of ^{14}C-butylated chloramphenicol/hour/100 μg of protein.

RNA Analysis. Total RNA from rat or mouse livers or from cultured rat primary hepatocytes was isolated using the guanidinium isothiocyanate procedure as mentioned previously (Chapter 2). The cDNA for CYP4A2 was generated by A. Thelen (Department of Physiology, MSU) by differential display screening. mRNA levels were measured by dot and Northern blot analyses and the level of hybridization was quantified using a Molecular Dynamics phosphoimager (Sunnyvale, CA) or by videodensitometry using an Agfa-2 scanner linked to a Macintosh computer with NIH Image software.

Statistical Analysis. All data are presented as the mean ± S.E. Statistical comparisons were made by a single-factor factorial analysis of variance using Microsoft Excel 5.0.

Results

The effects of olive and fish oil on hepatic gene expression in wild-type (+/+) and PPARα knockout (-/-) mice. PPARα is the predominant PPAR subtype in rodent liver and has a central role in regulating the transcription of genes encoding hepatic peroxisomal and microsomal enzymes (Lee et al., 1995; Braissant et al., 1996; Schoonjans et al., 1996). To determine whether PPARα mediates PUFA regulation of hepatic gene expression, wild type (+/+) and PPARα knockout (-/-) mice were fed an olive oil or fish oil diet for 5 days. Northern analyses show that feeding (+/+) mice fish oil for 5-days resulted in a ~2-fold ($p < 0.003$) and ~9-fold ($p < 0.001$) increase in hepatic $mRNA_{AOX}$ and $mRNA_{CYP4A2}$, respectively (Figure 22 and Table 2). In contrast, fish oil did not

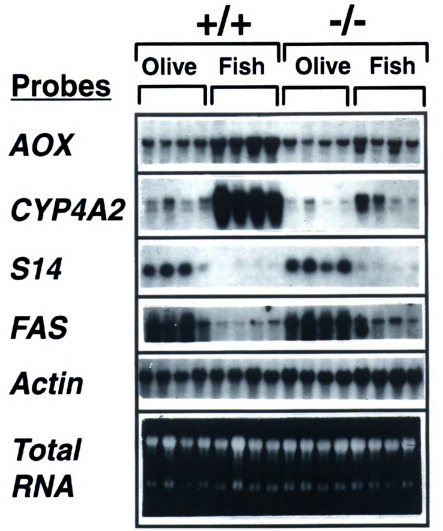


Figure 22 . Effects of fish oil feeding on mouse gene expression

Eight mice of each genotype were meal-fed with diets supplemented with 10% olive oil for 10 days. Four of each genotype were switched to a diet supplemented with 10% fish oil for 5 days. Total RNA was prepared from mouse livers and measured for mRNAs encoding AOX, CYP4A2, S14, FAS and β-actin by Northern analysis. The AOX, CYP4A2, S14 and FAS and β-actin blots were exposed to X-ray film for 36, 18, 24, 24, 18 hrs, respectively.

Table 2. Relative mRNA levels of mice fed on olive oil and fish oil

	Relative mRNA Abundance					
mRNA	Wild-type (+/+)			PPARα-knockout (-/-)		
	Olive oil	Fish oil	ANOVA	Olive oil	Fish oil	ANOVA
AOX	1 ± 0.1	2.3 ± 0.5	p<0.003	1.1 ± 0.2	1.4 ± 0.3	N.S.
CYP4A2	1 ± 0.3	8.9 ± 1.2	p<0.001	0.5 ± 0.3	1.3 ± 1.1	N. S.
S14	1 ± 0.2	0.05 ± 0.03	p<0.001	1.1 ± 0.2	0.23 ± 0.01	p<0.001
FAS	1 ± 0.4	0.2 ± 0.1	p<0.012	1.6 ± 0.4	0.4 ± 0.3	p<0.013
β-Actin	1 ± 0.2	1.3 ± 0.3	N. S.	1.7 ± 0.2	2.1 ± 0.5	N. S.

significantly induce $mRNA_{AOX}$ and $mRNA_{CYP4A2}$ in the PPARα knockout (-/-) mice. These results indicate that PPARα is required for the PUFA-mediated induction of AOX and CYP4A2 mRNAs. While hepatic β-actin mRNA was elevated in the (-/-) mice when compared to the (+/+) mice, it was not affected by dietary manipulation. Analysis of mRNAs encoding S14 and FAS shows both mRNAs were suppressed more than 70% in both the wild type (+/+) and PPARα knockout (-/-) mice following fish oil feeding. Since PUFA rapidly inhibits the transcription of both the S14 and the FAS gene (Jump et al., 1993, 1994), these observations indicate that PPARα is not required for the PUFA-mediated suppression of transcription of these genes.

The effects of olive oil and fish oil on hepatic acyl CoA oxidase (AOX) and S14 gene expression. Previous studies have shown that S14 and FAS are regulated by PUFA and peroxisomal proliferators in rat liver or primary hepatocytes (Jump et al., 1994, 1995). The studies described below will compare the PUFA and peroxisome proliferator regulation of S14 and AOX in rat liver and primary hepatocytes. These *in vivo* and *in vitro* (rat primary hepatocytes) studies were performed to gain additional support for the idea that PUFA regulation of lipogenic gene expression is a result of a different pathway other than PUFA regulation of AOX gene expression.

Rats were meal-fed diets supplemented with 10% olive oil, fish oil, eicosapentaenoic acid (20:5) or docosahexaenoic acid (22:6) for 5 days. When compared to chow-fed rats, hepatic $mRNA_{AOX}$ is elevated ~ 40% in olive oil-fed rats and ~ 3-fold in fish oil, 20:5 and 22:6-fed rats (Figure 23). $mRNA_{CYP4A2}$ was induced >10-fold by fish oil. While $mRNA_{S14}$ is induced ~ 2-fold by the olive oil feeding, fish oil, 20:5 and 22:6 suppressed $mRNA_{S14}$ by ≥ 78%. Hepatic $mRNA_{FAS}$ is also suppressed in fish oil-fed rats (Jump et al., 1994). Feeding mice (Figure 22) or rats (Figure 23) fish oil or their highly unsaturated fatty acid constituents (20:5,n-3 or 22:6,n-3) leads to a pronounced induction of $mRNA_{AOX}$ and $mRNA_{CYP4A2}$ while inhibiting expression of $mRNA_{S14}$ and $mRNA_{FAS}$.

Time course of fish oil and gemfibrozil effects on hepatic S14 and acyl CoA oxidase gene expression in vivo. The rapidity of fish oil action on hepatic $mRNA_{AOX}$ and $mRNA_{S14}$ was examined in rats fed fish oil for 1 to 5 days (Figure 24). Rats were meal-fed a high carbohydrate diet supplemented with 10% triolein oil for 10 days. Subsequently, half of the rats were maintained on this diet and half were switched to a high-carbohydrate diet supplemented with 10% fish oil. Fish oil feeding induced a rapid suppression of

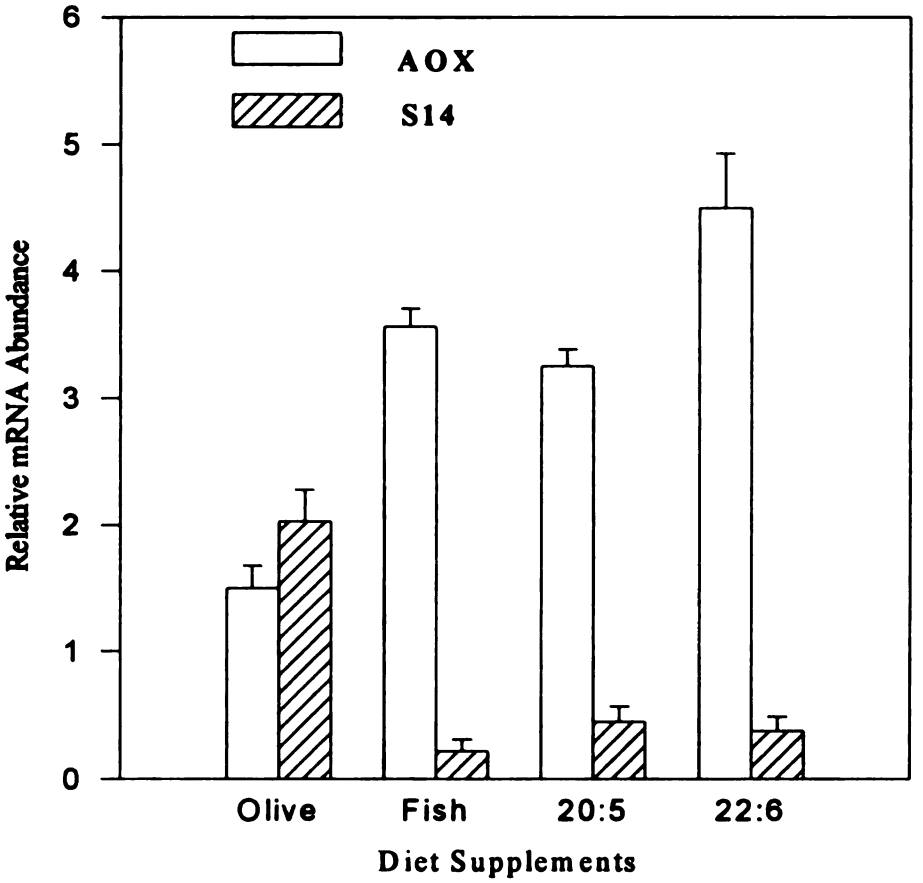


Figure 23. A comparison of the effect of olive oil and fish oil on hepatic S14 and AOX gene expression *in vivo*

Rats were meal-fed with diets supplemented with 10% (wt/wt) olive oil, menhaden (Fish) oil, eicosapentaenoic acid (20:5) or docosahexaenoic acid (22:6) for 5 days. Total hepatic RNA was prepared and examined by dot blot analysis for the effect of feeding on $mRNA_{AOX}$ (open bars) and $mRNA_{S14}$ (solid bars) levels. The results were quantified and normalized against the level of hepatic mRNA expressed in chow-fed rats, i.e., 1 unit. ANOVA: for both $mRNA_{AOX}$ and $mRNA_{S14}$ levels: menhaden oil; 20:5; 22:6 fed vs. olive oil fed, $P< 0.002$. . These results are representative of 2 separate studies; N=4.

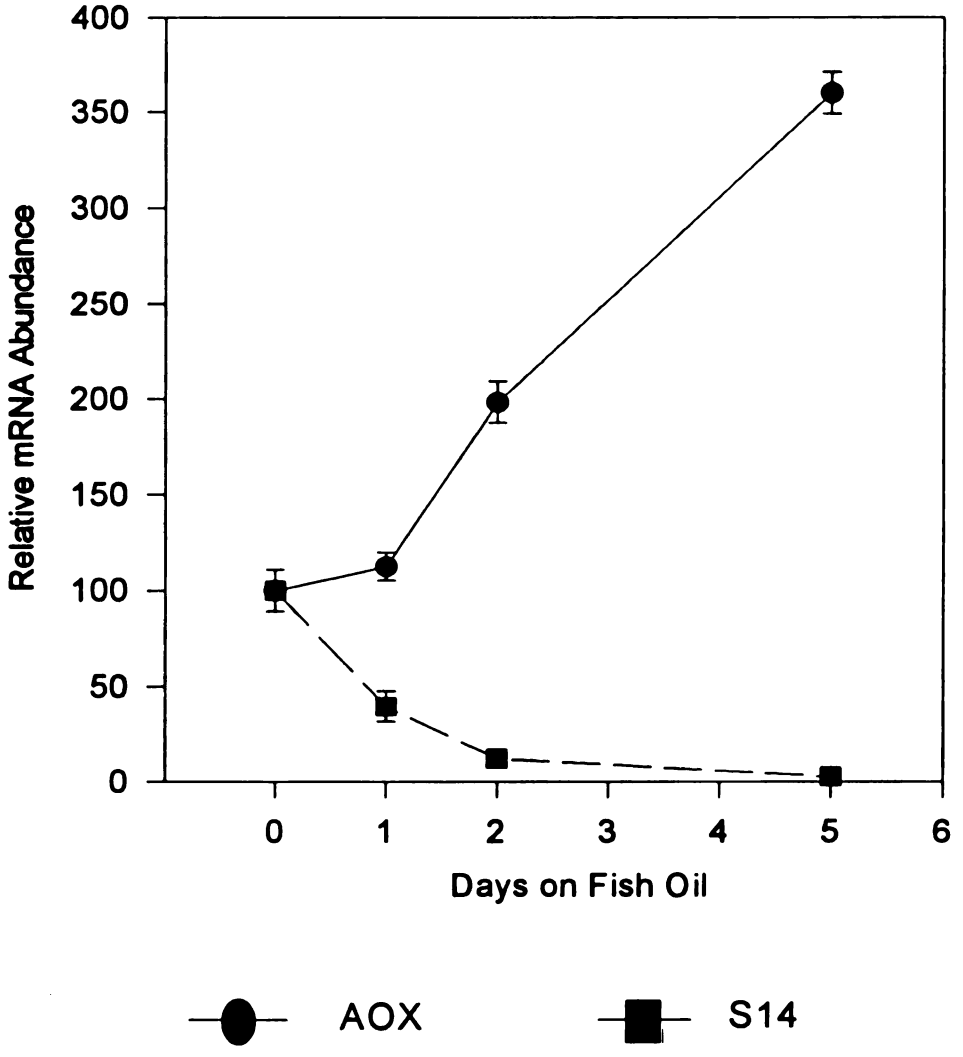


Figure 24. Time course of fish oil effects on rat hepatic S14 and acyl CoA oxidase gene expression

Rats were meal-fed diets supplemented with 10% (wt/wt) triolein oil for 10 days. Half of the rats were maintained on triolein oil while the other half were switched to the fish oil diet. Both triolein and fish oil-fed rats were killed 1, 2 and 5 days afterward. Total liver RNA was prepared and examined by dot blot analysis for $mRNA_{AOX}$ (circles) and $mRNA_{S14}$ (squares) levels, N = 4-10 per time point. These results are representative of 2 separate studies. The results were quantified and normalized against the level of hepatic mRNA expressed in meal-trained rats.

$mRNA_{S14}$: a 60% suppression was observed within 1 day of switching the diet from triolein to fish oil. Similar effects on mRNAs encoding FAS and L-pyruvate kinase have been reported previously (Jump et al., 1994). In contrast, $mRNA_{AOX}$ remained unaffected after 1 day on the fish oil diet, yet was induced 2-fold and 3.5-fold after 2 and 5 days, respectively. Such studies indicate that changes in S14 mRNA precede changes in AOX mRNA following initiation of fish oil feeding, but they do not argue against PPARα as a common mediator for the PUFA-regulation of AOX and S14.

In an effort to separate the induction of AOX from the suppression of S14, the peroxisome proliferator, gemfibrozil was fed to rats at 0.2% (wt/wt) for up to 8 days (Figure 25). $mRNA_{AOX}$ was induced ~4-fold after 4 days on gemfibrozil, a level comparable to the level of $mRNA_{AOX}$ after 5 days on fish oil. In contrast, gemfibrozil did not significantly suppress $mRNA_{S14}$ (Figure 25) or $mRNA_{FAS}$ (data not shown). Only a modest 22% inhibition of $mRNA_{S14}$ was seen after 4 days of gemfibrozil feeding. These results show that mRNAs encoding both S14 and AOX are affected by PUFA within 2 days of initiating fish oil feeding. However, the absence of a significant inhibition of $mRNA_{S14}$ following 8 days of gemfibrozil feeding argues against PPARα as a common mediator for PUFA regulation of both AOX and S14 gene expression.

Effect of fatty acids on AOX and S14 gene expression in primary hepatocytes. Primary hepatocytes provide a method to assess the direct effects of PUFA on hepatic gene expression (Jump et al., 1993, 1994). To examine the effects of fatty acids on S14 and AOX mRNAs, primary rat hepatocytes were treated with albumin alone or albumin plus various fatty acids (Figure 26). Treatment of primary hepatocytes with 18:1, 18:2, 18:3 (both n-3 and n-6) and 20:4 did not induce $mRNA_{AOX}$. Only 20:5 treatment

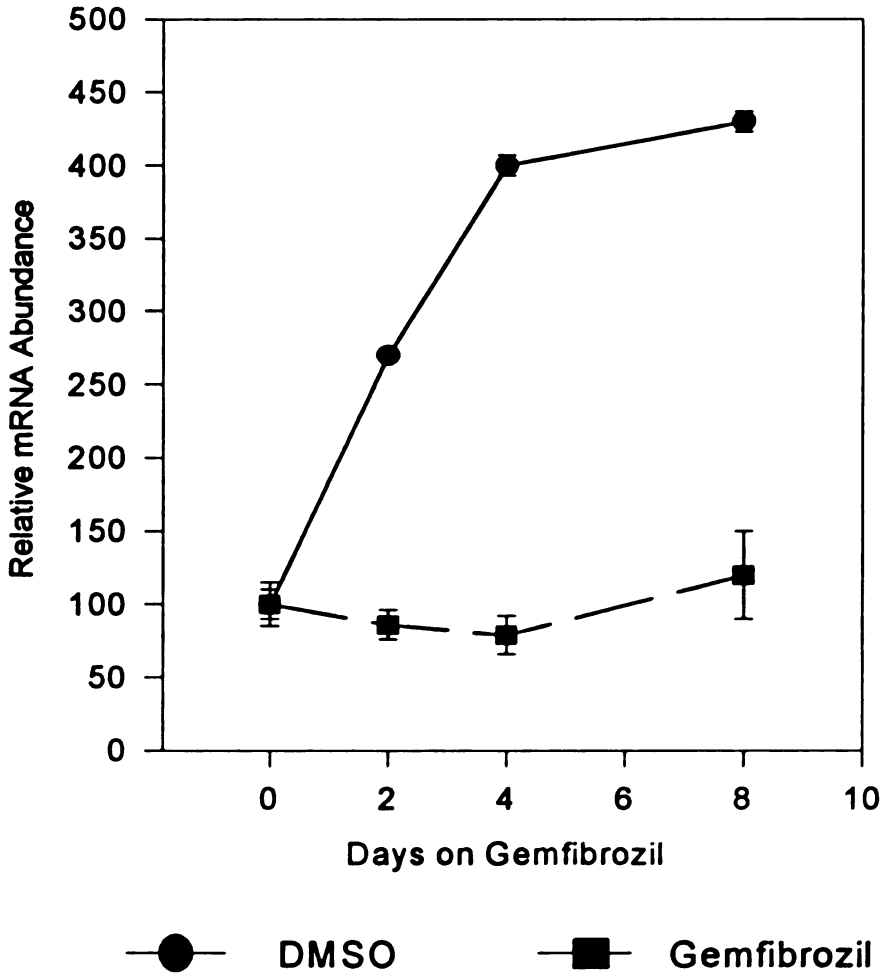


Figure 25. Time course of gemfibrozil effects on rat hepatic S14 and acyl CoA oxidase gene expression

Rats were meal-fed diets supplemented with 0.2% (wt/wt) gemfibrozil for 2, 4 and 8 days. Dot blot analysis was used to measure the $mRNA_{AOX}$ (circles) and $mRNA_{S14}$ (squares) levels. Total liver RNA was prepared and examined by dot blot analysis for $mRNA_{AOX}$ and $mRNA_{S14}$ levels, N = 4-10 per time point. These results are representative of 2 separate studies. The results were quantified and normalized against the level of hepatic mRNA expressed in meal-trained rats.

Figure 26. A comparison of various fatty acids on S14 and acyl CoA oxidase gene expression in primary rat hepatocytes

Primary rat hepatocytes prepared by the collagenase perfusion were plated into 6-well Primaria tissue culture dishes in the presence of 1μM T_3, 1μM insulin and 50 μM albumin. The cells were maintained in media containing different fatty acids (250 μM) for 48 hours (17). Total RNA was prepared from the hepatocytes and analyzed by dot blot analysis for mRNA coding for AOX and S14. The results were quantified and normalized against the level of AOX or S14 mRNA expressed in hepatocytes receiving no fatty acid treatment (Albumin Control). ANOVA: **a**: 18:1, n-9; 18:2, n-6; 18:3, n-3; 18:3, n-6 or 20:4, n-6 treated $mRNA_{AOX}$ vs. albumin treated $mRNA_{AOX}$, $P \geq 0.07$. **b:** 20:5, n-3 treated $mRNA_{AOX}$ vs. albumin treated $mRNA_{AOX}$, $P = 0.006$. **c**: 18:1 treated $mRNA_{S14}$ vs. albumin treated $mRNA_{S14}$, $P = 0.01$. **d**: 18:2, n-6; 18:3, n-3; 18:3, n-6; 20:4, n-6 or 20:5, n-3 treated $mRNA_{S14}$ vs. albumin treated $mRNA_{S14}$, $P \leq 0.0004$. These results are representative of 2 separate studies; N=4.

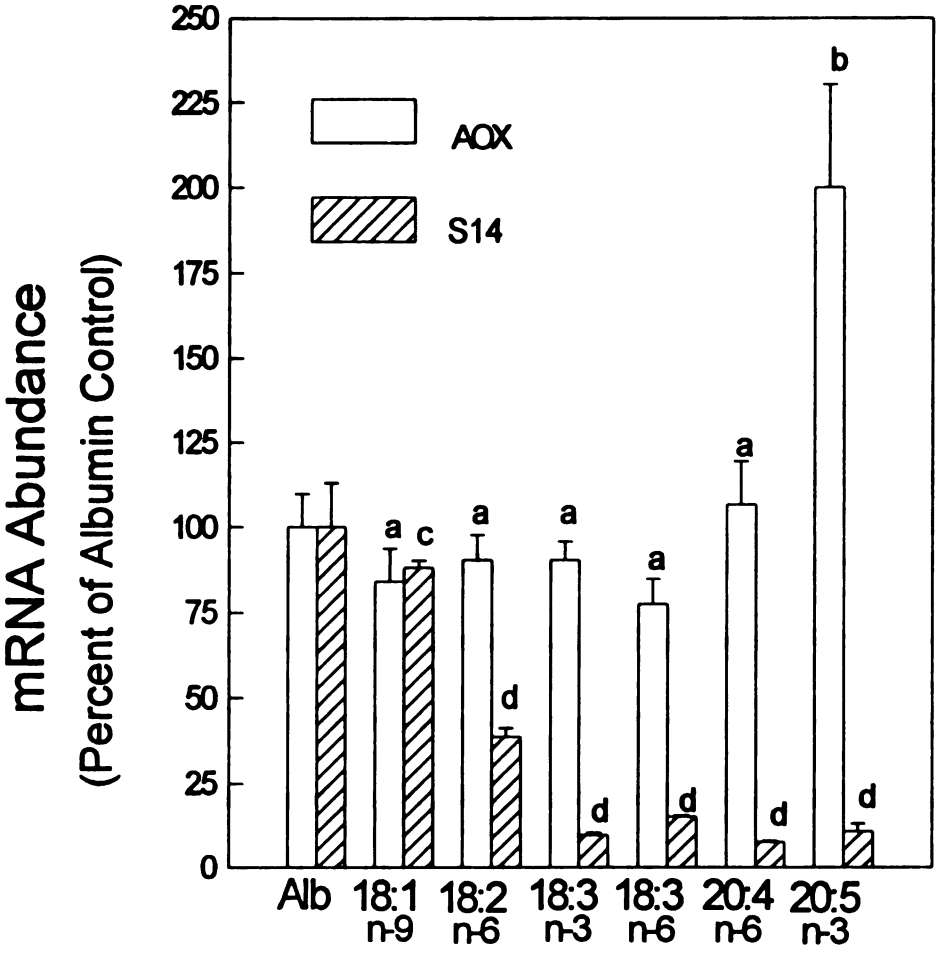

mRNA Abundance
(Percent of Albumin Control)
250
225
200
175
150
125
100
75
50
25
0
AOX
S14
a
c
a
d
a
d
a
d
b
d
Alb
18:1 n-9
18:2 n-6
18:3 n-3
18:3 n-6
20:4 n-6
20:5 n-3

Figure 26

induced $mRNA_{AOX}$ (~ 2-fold). This finding is consistent with the effects of highly unsaturated fatty acids on AOX gene expression *in vivo* (Figures 23 and 24). Oleic acid (18:1) did not affect S14 gene expression when compared to controls. However, 18:2 (n-6) resulted in ~50% decline and 18:3 (both n-3 and n-6), 20:4 (n-6) and 20:5 (n-3) treatment resulted in >80% suppression of hepatocyte $mRNA_{S14}$ levels. These findings demonstrate that a broader spectrum of fatty acids affect S14 gene expression than AOX gene expression in primary rat hepatocytes.

Fatty acid effects on reporter gene activity in primary hepatocytes. To determine if fatty acids activate PPARα in liver, primary hepatocytes were transfected with a reporter gene containing the AOX PPRE fused to the thymidine kinase promoter, i.e. TKCAT223 (Ren et al., 1996). Primary hepatocytes were cotransfected with pSG5 (empty vector) or pSG5-mPPARα, a PPARα expression vector (Figure 27). In the absence of cotransfected PPARα, TKCAT223 CAT activity was expressed at low levels (<150 CAT Units) and this activity was marginally affected by fatty acid or peroxisome proliferator (WY14,643, gemfibrozil) treatment. Cotransfection with pSG5-PPARα led to at least a 10-fold stimulation of the TKCAT223 activity. Treatment of PPARα-transfected hepatocytes with 18:1 and 20:4 had no effect on CAT activity, while 20:5 treatment induced CAT activity by ~2-fold. This pattern of fatty acid regulation of PPARα is consistent with the effects of these fatty acids on $mRNA_{AOX}$ (Figure 26). By comparison, both WY14,643 and gemfibrozil induced CAT activity by 4-fold. These studies show that long chain unsaturated fatty acids such as 18:1, 18:2, 18:3 (n-3 and n-6) and 20:4 do not activate PPARα in primary hepatocytes. Only the highly unsaturated fatty acid, 20:5, n-3 activates PPARα, albeit to a level less than Wy14,643 or gemfibrozil. This pattern of

Figure 27. Activation of PPARα by fatty acids and peroxisome proliferators

Primary hepatocytes cotransfected with TKCAT223 (1 μg) in the presence of 0.5 μg pSG5 (open bars) or pSG5-PPARα (closed bars). The cells were treated with different fatty acids at the concentration of 250 μM in the presence of 50 μM albumin. A second group of cells were treated with either 100 μM WY14,643 or 100 μM gemfibrozil. Media was replaced after 24 hours and cells were harvested after 48 hr of treatment and assayed for protein levels and CAT activity. ANOVA: **a**: Without PPARα, 18:1, 20:4 or 20:5 vs. albumin, $P \geq 0.3$. **b**: With PPARα, 18:1 or 20:4 vs. albumin, $P \geq 0.1$. **c**: With PPARα, 20:5 vs. albumin, $P = 0.04$. **d**: With or without PPARα, $P \leq 0.007$. These results are representative of at least 2 separate studies, N=3/study.

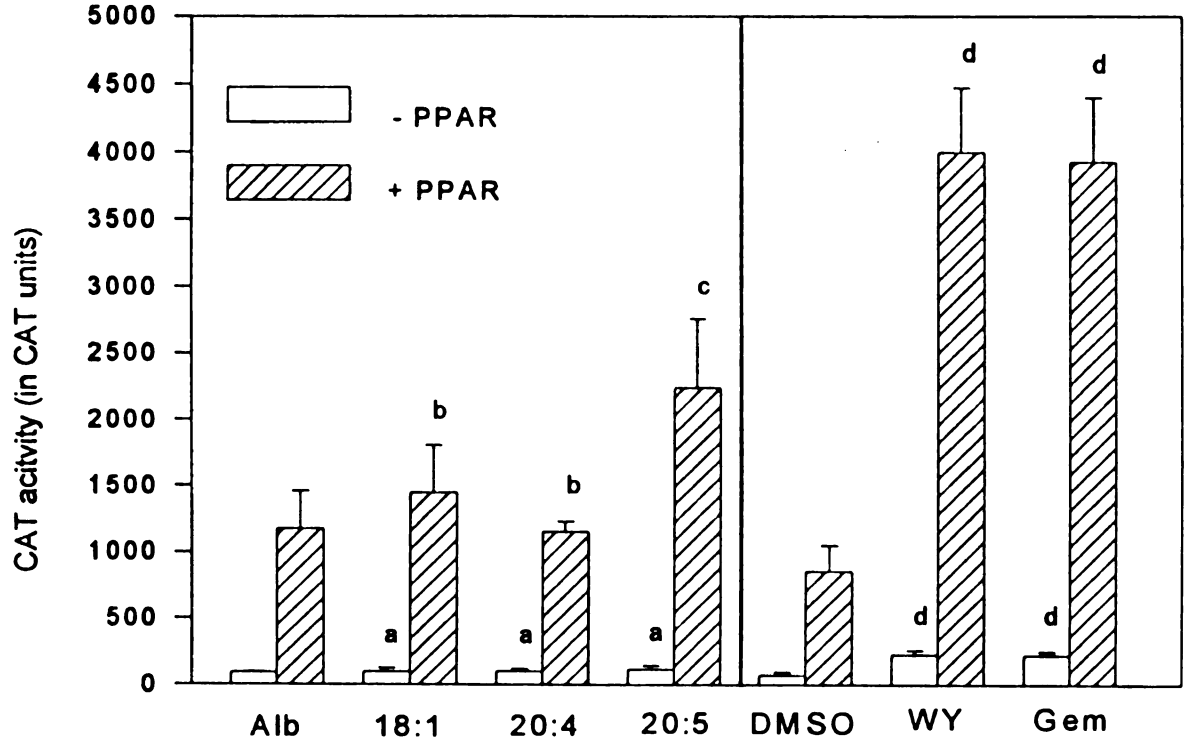

5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CAT acitvity (in CAT units)
- PPAR
+ PPAR
a
b
a
b
a
c
d
d
d
d
Alb
18:1
20:4
20:5
DMSO
WY
Gem

Figure 27

control contrasts with the known effects of fatty acids on $mRNA_{S14}$ (Figure 26, Jump et al., 1993, 1994) and S14CAT activity in primary hepatocytes where 18:2 (n-6), 18:3 (n-3 and n-6), 20:4 (n-6) and 20:5 (n-3) inhibit S14 gene expression 50-80%.

Discussion

PPARα is the predominant PPAR subtype expressed in rat liver and it plays a central role in the induction of hepatic peroxisomal and microsomal fatty acid oxidation (Lee et al., 1995; Braissant et al., 1996). Since several peroxisomal, microsomal and lipogenic enzymes are regulated by PUFA at the pretranslational level, I tested the hypothesis that dietary PUFA regulate hepatic fatty acid oxidation and *de novo* lipogenesis through a common mediator, i.e. PPARα. Interestingly, all studies reporting on fatty acid regulation of PPARα have been carried out by over expressing receptors in established cell lines. No studies have directly examined the role PPARα may have in fatty acid-regulated hepatic gene transcription. The PPARα-knockout mouse allows such an analysis. Coupling this genetic approach with other studies has allowed us to show for the first time that: 1) PPARα is required for PUFA-mediated induction of hepatic $mRNA_{AOX}$ and $mRNA_{CYP4A2}$ (Figure 22); 2) PPARα is not required for PUFA-mediated suppression of $mRNA_{S14}$ or $mRNA_{FAS}$ (Figure 22); 3) while 18:2 n-6, 18:3 (n-6 and n-3), 20:4 n-6 and 20:5 n-3 suppress $mRNA_{S14}$ and $mRNA_{FAS}$, only 20:5,n-3 induces $mRNA_{AOX}$ in primary hepatocytes (Figure 26); 4) while gemfibrozil induces hepatic $mRNA_{AOX}$, it has little or no effect on $mRNA_{S14}$ or $mRNA_{FAS}$ (Figure 25). Taken together, these studies indicate that PUFA control of peroxisome/microsomal fatty acid oxidation and *de novo* lipogenesis in rat liver does not involve PPARα as a common mediator. The differential effect of specific

fatty acids , i.e., 18:2, 18:3 n-3 and n-6, 20:4 n-6, versus gemfibrozil underscores the lack of coordinate regulation of these pathways in rat liver. Such studies indicate that PUFA regulates at least 2 pathways in liver. One involves PPARα and controls expression of genes encoding proteins involved in peroxisomal and microsomal fatty acid oxidation. The other mechanism is PPARα-independent and is involved in the PUFA-mediated suppression of lipogenic gene expression.

PUFA suppress hepatic $mRNA_{S14}$ and $mRNA_{FAS}$ levels by inhibiting gene transcription (Blake and Clarke, 1990; Jump et al., 1993, 1994). From the data reported above, this inhibitory mechanism does not require PPARα. Although the mechanism of PUFA induction of hepatic $mRNA_{AOX}$ and $mRNA_{CYP4A2}$ has not been established, the following studies implicate transcription as the principal mode of PUFA regulation of AOX and CYP4A: 1) peroxisomal proliferators rapidly induce transcription of genes encoding AOX, the bifunctional enzyme, thiolase and CYP4A subtypes 1-3 (Reddy and Mannaerts, 1994; Lee et al., 1995); 2) PPARα is required for the induction of these genes (Lee et al., 1995); 3) PPARα binds PPREs as PPAR/RXR heterodimers in the promoters of these genes and stimulates transcription of cis-linked reporter genes (Kliewer et al., 1992; Gearing et al., 1994; Keller et al, 1995; Ren et al., 1996); 4) fatty acids activate PPARα and stimulate transcription of cis-linked reporter genes (Figures 26, 27); 5) PPARα is required for the PUFA induction hepatic $mRNA_{AOX}$ and $mRNA_{CYP4A2}$ (Figure 22).

Previous efforts to examine the involvement of PPARα in PUFA regulation of lipogenic gene expression showed that the cis-regulatory targets for PUFA and PPAR in the S14 promoter (Jump et al., 1995; Ren et al., 1996) promoter did not converge.

Analysis of stearoyl CoA desaturase 1 gene expression indicated that peroxisome proliferators/PPAR induced by PUFA suppressed transcription (Miller and Ntambi, 1996). Such studies argued against PPAR as a mediator of PUFA effects on lipogenic gene transcription. However, the overexpression of receptors does not necessarily reflect physiologically relevant processes. The use of the PPARα-knockout mouse allows us to directly evaluate the role PPARα plays in PUFA regulation of hepatic gene expression. In contrast to (+/+) mice, hepatic $mRNA_{AOX}$ and $mRNA_{CYP4A2}$ was not significantly induced in PPARα (-/-) mice by the PUFA diet indicating a requirement for PPARα in the PUFA-mediated induction of these enzymes. The fact that hepatic $mRNA_{S14}$ and $mRNA_{FAS}$ was suppressed in both (+/+) and (-/-) mice provides strong evidence against a requirement for PPARα for PUFA-mediated suppression of S14 and FAS gene transcription. While these studies confirm our earlier suggestion that PPAR did not mediate PUFA suppression of S14 gene transcription, they provide new information on the requirement for PPARα in the PUFA-induction of AOX and CYP4A2 and the lack of involvement of PPARα in PUFA-mediated suppression of FAS gene transcription or L-pyruvate kinase gene expression. While other PPAR subtypes [PPARγ and PPARδ] (Braissant et al., 1996; Huang et al., 1994) are expressed in liver, northern analyses suggests PPAR γ and δ are minor subtypes in rodent liver. However, their role in PUFA control of hepatic gene expression cannot be excluded.

An important outcome of these studies is the finding that of all the PUFA tested, only 20:5 n-3 activates PPARα in liver. Several groups have reported on fatty acid activation of PPARs in established cells lines like CV-1 and HeLa (Gottlicher et al., 1992; Keller et al., 1993; Kliewer et al., 1994; Schoonjans et al., 1996; Braissant et al., 1996).

Recently 3 groups reported that specific fatty acids, i.e., 18:2 n-6, 18:3 (n-3 and n-6) and 20:4 n-6 are ligands for PPARα (Forman et al., 1997; Kliewer et al., 1997; Krey et al., 1997). These same ligands do not activate PPARα or induce $mRNA_{AOX}$ in primary hepatocytes (Figures 25 and 26). Feeding animals soybean or corn oil, oils containing 18:2 and 18:3 fatty acids, does not induce peroxisomal enzymes (Flatmark et al., 1988). This apparent conflict can be reconciled by the fact that primary hepatocytes have a high capacity for fatty acid oxidation, triglyceride synthesis and VLDL secretion (Rustan et al., 1988). We speculate that these pathways prevent intracellular fatty acids from accumulating to levels that are high enough to activate PPARα. Interestingly, 20:5, n-3 was the only PUFA tested here that activated PPARα. 20:5 n-3 is reported to be poorly oxidized in mitochondria and poorly incorporated into complex lipids, such as triglycerides (Rustan et al., 1988). Thus, 20:5,n-3 might accumulate in the cell and mimic a state of fatty acid overload in the liver. Fatty acid overload resulting from high fat feeding [>50% calories as fat], uncompensated diabetes and liver disease have all been reported to increase peroxisomal β-oxidation (Reddy and Mannaerts, 1994; Kaikaus et al., 1993). Alternatively, 20:5,n-3 might be metabolized to an active ligand. Recent studies have suggested that the leukotriene, LTB4, is a ligand for PPARα (Devchand et al., 1996). Indeed, LTB4 is derived from 20:4,n-6 by the action of 5-lipoxygenase and LTA4 hydrolase. If this pathway were operative, we would expect 20:4, n-6 treatment of hepatocytes to activate PPARα and induce $mRNA_{AOX}$. The lack of a 20:4,n-6 effect on $mRNA_{AOX}$ and PPARα along with the low LTA4 hydrolase activity associated with liver cells (Yokomizo et al., 1995) suggest that LTB4 is not the operative ligand for 20:5,n-3

activation of PPARα. However, the *in vitro* model used in this work may lack factors present in the *in vivo* system.

In summary, PUFA induce peroxisomal and microsomal fatty acid oxidation and suppress *de novo* lipogenesis. This apparent coordinate regulation of lipid metabolism does not involve PPARα as a common mediator. While highly unsaturated n-3 fatty acids, like 20:5,n-3 can activate PPARα resulting in increased $mRNA_{AOX}$ and $mRNA_{CYP4A2}$, PPARα does not mediate the suppressive effects of 18:2, 18:3 (n-3 and n-6), 20:4,n-6 or 20:5,n-3 on lipogenic gene expression. Thus, PUFA suppression of S14 and FAS gene transcription is mediated by a pathway that is independent of PPARα. The underlying mechanism of this alternative pathway is currently under investigation.

Chapter 5. Summary and Conclusions

My dissertation project was designed to investigate the mechanism of PUFA inhibition of hepatic S14 gene transcription. To solve the issue, the first step I chose was to identify the molecular mediator of PUFA action. The similar inductory effects of long-chain PUFA and peroxisome proliferators on AOX gene expression led to a hypothesis that PPAR might mediate the PUFA inhibition of S14 gene expression. Therefore I started by examining the candidacy of PPAR as PUFA-RF.

Treatment of hepatocytes with Wy14,643, a potent activator of PPARα, showed dramatic inhibition of the S14 mRNA level and S14CAT activity. The similar extent of inhibition of both S14 mRNA abundance and S14 promoter activity suggested the level of inhibition is at the pre-translational level. However, based on the results shown in this thesis, the possibilities that Wy14,643 affects S14 mRNA splicing or stability can not be totally ruled out.

To our surprise, the target of Wy14,643 inhibition of S14 gene transcripiton is the S14 TRR, instead of S14 PUFA-RE. The functional and spacial distinctiveness of the nPPRE and nPUFA-RE led us to the conclusion that PPARα does not mediate the PUFA inhibition of S14 gene transcription. By gel-shift analysis and transfection studies, I demonstrated that the mechanism of PPAR inhibition of S14 gene transcription is through a mechanism involving receptor sequestration. PPARα competes with TRβ for the limited amount of RXR in hepatocytes. As a result of this competition, the T_3 induction of S14 gene transcription is suppressed in the presence of either peroxisome proliferator Wy14,643 or cotransfected PPAR. Therefore, the pathways regulated by peroxisome

proliferators, represented by Wy14,643, and by PUFA do not converge on the same *cis*-regulating elements controlling S14 gene transcription.

The last part of my study focused on examining how these two pathways, regulated by peroxisome proliferators and PUFA, control lipogenic peroxisomal and microsomal genes. Kinetic studies revealed that the PUFA inhibition of S14 mRNA precedes the elevation of AOX mRNA. A peroxisome proliferator, i.e. gemfibrozil, failed to suppress S14 mRNA in feeding studies. PPARα knockout mice feeding studies further confirmed that PPARα is not required for PUFA inhibition of S14 transcription. Therefore, in terms of inhibition of hepatic S14 gene, two pathways exist. One is mediated by PPAR, which targets the S14 TRR, and the other is mediated by the unknown PUFA-RF, which targets S14 PUFA-RR in the proximal promoter.

In other gene models, it appears that there are some crosstalk between the two pathways. As demonstrated by fish oil feeding PPAR knockout mice studies, fatty acid activate AOX and CYP gene expression via PPARα. In cultured hepatocytes, 18:2, 18:3 (n-3 and n-6) and 20:4 all failed to stimulate AOX gene expression, measured by both AOX mRNA and PPRE-CAT activity. Only 20:5, which is abundant in fish oil, is able to elevate the AOX mRNA level and activate PPAR.

Dietary fatty acids may regulate transcription factor function by at least two general mechanisms. One involves binding of an activating ligand by the transcription factor. The other involves the regulation of transcription factor through covalent modification, such as phosphorylation, redox state or covalent modification. I have focused on the former pathway and excluded PPARα as a putative PUFA-RF. Future

effort may be directed to investigate other possible mechanisms of PUFA inhibition of S14 transcription.

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