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# COMMENTARY

# RECEPTOR-MEDIATED MECHANISMS OF PEROXISOME PROLIFERATORS

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## *Fibrate hypolipidemic drugs*

Coronary heart disease represents a major health problem. Regimens, such as adopting a controlled diet or the administration of drugs to reduce the level of atherogenic lipids associated with an increased risk of coronary heart disease, are therefore likely to be beneficial in decreasing mortality from cardiovascular disease [1, 2]. The fibrate class of hypolipidemic drugs was discovered some 30 years ago and represents a first generation of drugs capable of lowering serum triglyceride and cholesterol levels [3]. One reason for the blunted optimism surrounding the clinical use of such drugs is the observation that, where tested, fibrate hypolipidemic drugs are rodent hepatocarcinogens [4, 5]. This has led to a greater interest in other lipid-lowering drugs such as nicotinic acid, agents that sequester bile acids, and inhibitors of cholesterol synthesis [6, 7].

In humans, clofibrate is more effective at reducing the levels of triglycerides (VLDL†) than cholesterol (LDL), and the drug could therefore be effective in the treatment of type IV hyperlipidaemia [8]. Clinical trials using clofibrate have demonstrated a reduced incidence of non-fatal myocardial infarction but no effect on mortality due to coronary heart disease [9]. Trials using other fibrates, such as gemfibrozil, indicate them to be well tolerated and effective hypolipidemic drugs in patients with severe hypertriglyceridemia [10, 11]. The mechanism of action of fibrates such as clofibrate [3] is unknown. Early research in the rat suggested that clofibrate acted by displacing androsterone and thyroxine from serum albumin [3, 12], since both have some hypolipidemic effects [3, 13, 14]. However, new research now suggests that the action of fibrate drugs could be mediated by specific receptor-mediated pathways (see below). One possible mechanism for the hypotriglyceridemic action of clofibrate is through an increase in lipoprotein lipase that hydrolyses VLDL triglycerides. This is supported by the ineffectiveness of clofibrate on serum triglycerides in patients with a genetically determined deficiency of lipoprotein lipase [15]. Other possible mechanisms include a decrease in cholesterol synthesis by reduction of hydroxy methyl glutaryl coenzyme A (HMG CoA) reductase [16] and the increased excretion of cholesterol through increased conversion to bile acids [15].

## *Peroxisome proliferation*

Clofibrate is one of many structurally diverse chemicals (Fig. 1) that include several hypolipidemic drugs, herbicides, leukotriene antagonists and plasticizers that together are termed peroxisome proliferators (PPs) [for reviews see Refs. 17–19]. Some of the more potent PPs are hypolipidemic drugs (e.g. Wy 14,643) whereas plasticizers such as di-(2-ethyhexyl) phthalate (DEHP) are much weaker. In rodents these compounds produce hepatomegaly as a result of both liver hyperplasia [20–22] and an increase in both the size and number of peroxisomes [20, 23]. Since natural factors such as a high-fat diet [24, 25] and the steroid dehydroepiandrosterone (DHEA) [26] can also induce peroxisome proliferation, it is probable that the phenomenon represents a physiological response to some natural biological stimulus.

The levels of several peroxisomal enzymes [23, 27], carnitine acetyl transferase [28] as well as members of the microsomal cytochrome P450 IV gene family [29] are elevated 10- to 30-fold in response to PP administration. The peroxisomal enzymes studied in the most detail include acyl CoA oxidase (ACO), bifunctional enzyme (BFE) and thiolase. Together, these enzymes are responsible for the peroxisomal $\beta$-oxidation of long chain fatty acids. ACO is the key enzyme in this pathway since it is both the rate-limiting step and, in addition, produces the hydrogen peroxide that some have implicated in the hepatocarcinogenic process (see below). All three inducible members of the P450 IVA family (IVA1, IVA2 and IVA3) have $\omega$-hydroxylase activity [30, 31]. IVA1 and IVA3 share 72% amino

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† Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HMG CoA, hydroxy methyl glutaryl coenzyme A; PP, peroxisome proliferator; DHEA, dehydroepiandrosterone; ACO, acyl CoA oxidase; BFE, bifunctional enzyme; DEHP, di-(2-ethylhexyl) phthalate; MEHP, mono(2-ethylhexyl)phthalate; DEN, diethyl-nitrosamine; PPAR, peroxisome proliferator activated receptor: PPBP, peroxisome proliferator binding protein; HRE, Hormone Response Element; ER, oestrogen receptor; GR, glucocorticoid receptor; TCA, trichloroacetic acid; RAR, retinoic acid receptor; TR, thyroid hormone receptor; VDR, vitamin $D_3$ receptor; THCA, 3,7,12-trihydroxycoprostanoic acid; TSH, thyroid stimulating hormone; CRABP, cellular retinoic acid binding protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and PPRE, peroxisome proliferator response element.

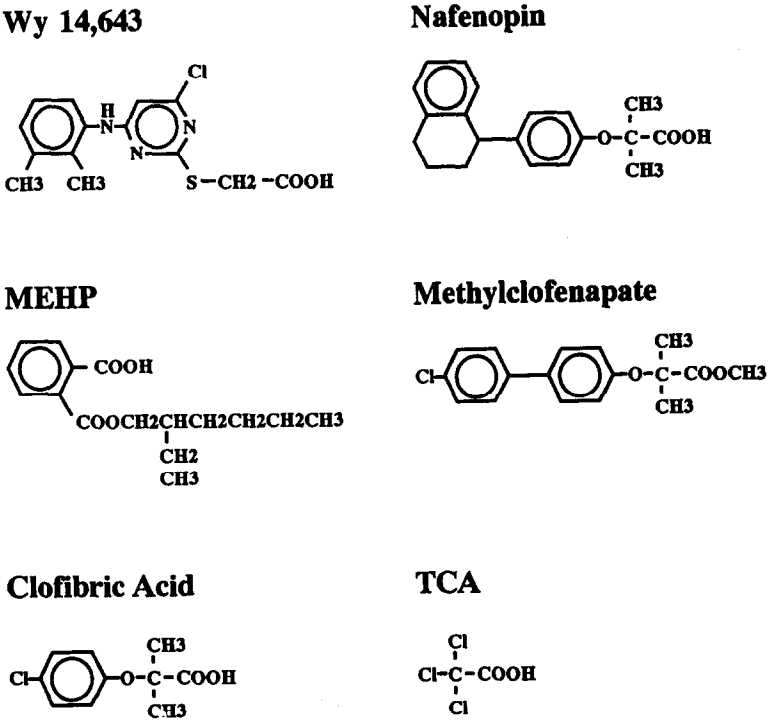


Fig. 1. Structures of selected peroxisome proliferators. Wy 14,643, nafenopin, methylclofenapate, and clofibric acid are all hypolipidemic drugs. MEHP is the primary metabolite of the plasticizer DEHP. The weak peroxisome proliferator, trichloroacetic acid (TCA), is a metabolite of solvents such as trichloroethylene [see Ref. 95].

acid identity and use a variety of substrates including fatty acids and prostaglandins $E_1$ and $F_{2\alpha}$ [31].

Induction of the $\beta$-oxidation enzymes [32, 33] and P450 IVA1 [34–36] is parallelled by an increase in their respective mRNAs. Increases are observed as early as 2 hr after PP administration, and this is partly, if not entirely, due to an increase in the transcription of the respective genes [32, 34, 37]. The apparent coordinate and rapid increase in expression of these genes may suggest a common mechanism of induction.

*Mechanism of hepatocarcinogenesis*

The basic mechanism(s) by which PPs induce tumours in rodents is unknown. PPs are termed non-genotoxic carcinogens since they fail to directly cause DNA damage when tested using a number of genotoxic assays, for example, the Ames *Salmonella* mutagenesis assay [38]. Moreover, the carcinogenic potencies of the various PPs differ considerably. For example, rats fed a diet containing 0.1% Wy 14,643 had a 100% liver tumour incidence after 60 weeks [39, 40] compared with only about 10% in male rats fed 1.2% DEHP for 2 years [41].

The oxidative stress hypothesis [17] is based upon a correlation between the ability of a compound to stimulate peroxisome proliferation and induce tumours. It is proposed that hydrogen peroxide, produced by an increase in peroxisomal fatty acid $\beta$-oxidation, results in oxidative stress leading to DNA damage and possibly tumour initiation [17]. This hypothesis is supported by a number of observations. First, there is an approximate doubling in the number of 8-OH-deoxyguanosine lesions in the liver of rats chronically fed a diet containing a potent PP [42] and DNA alterations are detectable by the $^{32}P$ post-labelling assay [43]. Second, there is a correlation between the potency of weak (DEHP) and potent (Wy 14,643) carcinogens and the level of accumulated hepatic lipofuscin used as an indicator of oxidative damage [44]. Third, the co-administration of PP with antioxidants reduced tumour incidence [45]. However, a comparison between the effects of DEHP and Wy 14,643 over a 12-month period indicated that tumours were observed in rats fed with Wy 14,643 but not with DEHP despite the fact that DEHP produced only 25% less peroxisome proliferation than Wy 14,643 [40]. Therefore, because of the better correlation between the carcinogenicity of DEHP and Wy 14,643 and their mitogenic effects in the liver it has been suggested that PPs could act as tumour promoters [40]. This is also supported by initiation–promotion protocols that demonstrate that Wy 14,643 increases the number of liver tumours in rat given an initiating dose of the genotoxic carcinogen diethylnitrosamine (DEN) [46] but fail to demonstrate any initiating activity of PPs [47].

A further intriguing insight into the hepatocarcinogenic mechanism of PPs comes from studies comparing the tumour promoting effects of phenobarbital and Wy 14,643 [46]. Here it was found that in rats given an initiating dose of DEN the action of

Wy 14,643 was to rapidly increase the size of altered foci whereas phenobarbital increased their number. This further suggests that the growth and differentiation effects of PPs are important in the carcinogenic mechanism.

Since PPs act as complete carcinogens in animal models, it is possible that they are able to act both as liver tumour initiators via oxidative stress and as liver tumour promoters via effects of growth and differentiation. Further work is required, however, to elucidate their true role in the carcinogenic process.

*Nuclear hormone receptor*

We and others have speculated upon the existence of specific receptors that could mediate the action of PPs [48, 49]. Reddy's group originally proposed the existence of a PP binding protein (PPBP) in rat liver [50, 51]. This 70 kDa binding protein was purified from rat liver on a nafenopin affinity column and the antibodies raised against it were used to identify cDNA clones [52]. The sequence of these clones indicates that PPBP is related to the heat shock protein hsp72. It should be noted, however, that direct binding of nafenopin to the expressed product of this clone has yet to be demonstrated and that PPBP is ubiquitously expressed at high levels making it unlikely to be a mediator of PP action. Furthermore, the potent PP Wy 14,643 does not bind to PPBP [51]. The possibility that the true biological mediator of PP action could be a member of the steroid hormone receptor superfamily prompted us to screen a mouse liver cDNA library using a probe derived from the combined sequences of several such receptors. This led to the identification of four new members of the hormone receptor family [48, 53]. Importantly, one of these receptors could be activated by the addition of a variety of PPs including hypolipidemic drugs and a plasticizer [48]. We have termed this receptor PPAR for the PP activated receptor.

The nuclear hormone receptor superfamily is comprised of at least 25 mammalian genes [for reviews see Refs. 54–56]. Some of these encode receptors for the classical steroid hormones and others bind thyroid hormones, vitamin $D_3$ and retinoic acid. However, the putative ligands for many remain to be identified, and such receptors have therefore been termed orphan receptors. Nuclear hormone receptors are intracellular proteins that bind their cognate ligand with high affinity and specificity. Additionally, they are DNA binding proteins that recognize short DNA motifs generally termed Hormone Response Elements (HREs). Such HREs are usually located upstream of the target genes and behave as transcriptional enhancers [57, 58]. The binding of the ligand–receptor complex to their cognate HRE can activate specific gene transcription. In some instances, the same receptor can behave as a transcriptional silencer and this appears to depend upon the context of the HRE within the promoter of each target gene [59–62]. Nuclear hormone receptors are therefore ligand-dependent transcriptional modulators.

The cloning of the cDNAs for these receptors has revealed that they all share a common primary organization with a highly conserved DNA and ligand binding domain (Fig. 2). The DNA binding domain is approximatley 70 amino acids in length and a comparison among the different members of the family reveals about 50% amino acid identity. The region contains a number of highly conserved amino acids and the tertiary structure is stabilized by two zinc atoms each binding to four invarient cysteine residues. This zinc stabilized structure is similar yet clearly distinct from the "zinc finger" DNA binding motif typified by the 5S ribosomal transcription factor TFIIIA [63]. Recent NMR [64] and crystallographic data have indicated that the receptor DNA binding domain folds into a single domain containing two alpha helical regions and hydrophobic core. There is good evidence indicating that amino acids within the first helix (the proximal box) make specific contacts within the major groove of DNA and therefore define target gene specificity [65–67].

The ligand binding domain is much larger being approximately 200–250 amino acids in length. It is speculated to fold to form a hydrophobic pocket that provides ligand binding specificity [68]. Beside binding ligand this domain contains regions important for transcriptional activation and in some cases receptor dimerization [69] and interaction with the heat shock protein hsp90 [70, 71]. Current opinion suggests that upon binding the correct ligand the ligand binding domain adopts an altered conformation. Where appropriate, this results in dissociation of hsp90 and dimerization (e.g. steroid hormone receptors) and the formation of a functional transcription activation domain capable of interacting in some way with the basic transcription machinery [54, 56].

*Activation of PPAR by peroxisome proliferators*

Chimeric receptors constructed using the DNA binding domain of either the oestrogen (ER-PPAR) or glucocorticoid (GR-PPAR) receptor and the putative ligand binding domain of PPAR are able to activate, respectively, an oestrogen- or glucocorticoid-responsive gene in the presence of PPs [48]. When the ER-PPAR was tested using several diverse PPs, a good correlation was observed between their ability to activate ER-PPAR and their potency either as PPs or as rat liver carcinogens (Fig. 2). For example, Wy 14,643 was more potent in the chimeric receptor assay than mono(2-ethylhexyl)phthalate (MEHP), the primary DEHP metabolite. These data suggest that PPAR could mediate the biological effects of PPs. This is further supported by the tissue specific expression of PPAR. The highest levels of expression are observed in the liver [48] which notably is also the tissue which shows the greatest response to PPs [72] and the highest incidence of tumours [17]. PPAR is also expressed in brown adipose tissue, kidney, heart and weakly in skeletal muscle, small intestine, testis and thymus ([48] and our unpublished results). This pattern of expression compares well with the tissue specific induction of ACO by PPs [72].

Given the diversity of PPs that activate PPAR, it is interesting to speculate whether they bind to PPAR directly or modulate its activity through some indirect mechanism. It has been proposed that PPs act by inhibiting mitochondrial metabolism leading

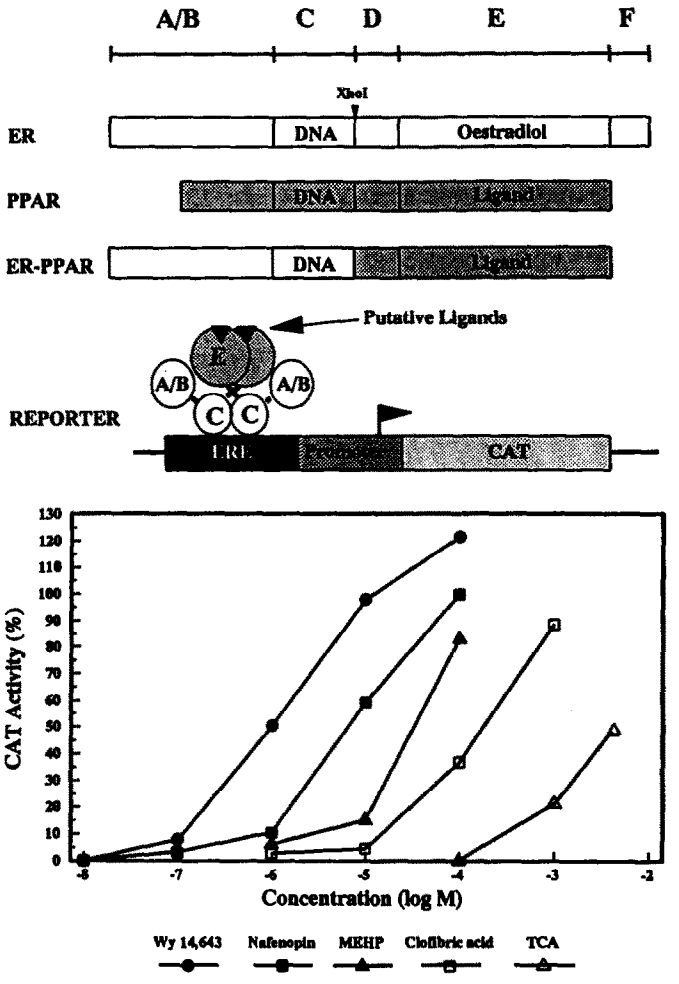


Fig. 2. Chimeric receptor trans-activation assay used to test peroxisome proliferators. The oestrogen receptor (ER) is shown divided into regions A–F based upon conserved receptor sequences. A unique restriction endonuclease site (XhoI) was created in both the ER and PPAR by site-directed mutagenesis to facilitate creation of the chimeric receptor cDNA ER-PAR. This plasmid was transiently expressed in mammalian cell lines to produce a chimeric receptor that binds to a reporter gene containing an oestrogen response element (ERE) and activates gene transcription in the presence of the appropriate ligand. The level of transcription is indirectly measured by assaying for chloramphenicol acetyl transferase (CAT) activity. Several peroxisome proliferators were tested, and CAT activity was expressed as a percentage of that observed using 100 $\mu$M nafenopin.

to an accumulation of fatty acids and the induction of P450 IV [18, 73]. It is possible that accumulation of a metabolite resulting from this type of lipid perturbation could be the natural ligand for PPAR. Alternatively, from an examination of the PPs that activate ER-PPAR (Fig. 1) it is conceivable that some of them could bind directly, although it is more difficult to imagine how trichloroacetic acid (TCA) could bind sufficiently well to allow transcriptional activation. Experiments using labelled nafenopin have so far failed to demonstrate any direct binding [48]. This could, however, be due to several factors such as the presumed low affinity of nafenopin for PPAR (as judged from the dose–response curves), from the low level of PPAR expression, or because of the presence of additional PP binding proteins such as PPBP [52]. Others, looking at enzyme induction in cultured hepatoctytes, have compared the activity of several PPs [74, 75] including a series of structurally related analogues of the leukotriene antagonist LY 171883 [76]. By comparing the structure–activity relationships of these chemicals, these studies underline the importance of an acid group and provide supportive evidence for direct interaction between PPs and some macromolecule, possibly PPAR.

It is probable that a natural ligand for PPAR exists. Given the similarity between PPAR and other

nuclear hormone receptors [48] such as the retinoic acid receptor (RAR), thyroid hormone receptor (TR) and vitamin $D_3$ receptor (VDR), it is difficult to know if the putative PPAR ligand will be steroid-like (e.g. like vitamin $D_3$) or not (e.g. thyroid hormones and retinoic acid). Peroxisomes are important in the metabolism of long chain fatty acids and the production of cholic acid from cholesterol [77]. Potential roles for such a ligand could therefore include the control of fatty acid or cholesterol metabolism. Possibly, therefore, the natural inducer of peroxisome proliferation may be a steroid, fatty acid or cholesterol metabolite. We have examined two potential candidate ligands, dehydroepiandrosterone (DHEA) and 3,7,12-trihydroxy-coprostanoic acid (THCA). DHEA is a steroid with hypolipidemic effects [78] that produces some peroxisome proliferation in rats [26]. Neither DHEA nor DHEA-sulfate when tested at 10 $\mu$M had any effect on ER-PPAR activation. However, more recently it has been reported that DHEA is only a PP *in vivo* and not *in vitro* [37]. Therefore, DHEA may need to be metabolized to become a potent PP or may perturb the natural metabolism of the animal to produce a proximate PP. THCA is a key cholesterol metabolite that undergoes $\beta$-oxidation exclusively in the peroxisomes to produce cholic acid [77]. In this case, too, 10 $\mu$M THCA was unable to activate ER-PPAR. Furthermore, evidence suggests that PPs do not stimulate the peroxisomal $\beta$-oxidation enzymes required to metabolize THCA [79]. Evidently, the search for the natural PPAR ligand will require a more pragmatic approach, but its identification could yield valuable information concerning the role of PPAR and its link with cancer.

*Does PPAR mediate peroxisome proliferator action?*

To define a link between the activation of PPAR and the phenomena of peroxisome proliferation and hepatocarcinogenesis, it will be important to determine whether PPAR is directly responsible for the transcriptional activation of PP responsive genes. Candidate genes include those for the $\beta$-oxidation enzymes and P450 IVA1 (see above). Of special interest is the peroxisomal $\beta$-oxidation enzyme ACO since this enzyme is responsible for the production of hydrogen peroxide and is therefore tentatively linked with cancer. If Reddy's oxidative stress hypothesis of PP-induced carcinogenesis (see above) proved to be correct and PPAR regulated the transcription of the ACO gene directly, then this would provide a direct link with receptor activation and both peroxisome proliferation and cancer.

A comparison of the primary amino acid sequence of PPAR with other nuclear hormone receptors reveals complete identity (TR, RAR, VDR) or a one amino acid difference (ER) within the proximal box of the DNA binding domain [48]. Since this region determines primary DNA binding specificity and each of these receptors binds to a DNA sequence related to TGACCT or TGAACT [64–67, 80, 81], then we would predict that PPAR also recognizes a similar motif. Examination of the promoter region of the rat ACO gene [82] reveals several such motifs and preliminary evidence suggests that one or more such motifs positioned approximately 570 nucleotides upstream of the transcription initiation start site are important in mediating the response to PPAR (our unpublished results) and PP [83]. More recent characterization of this PP response element (PPRE) has demonstrated that PPAR binds directly to these sequences (our unpublished results).

Interestingly the similarity of the proposed PPRE and those of other nuclear hormone receptors could indicate some degree of overlap between the gene networks regulated by PPs and those of, for example, retinoids and thyroid hormones. It is therefore of interest that PPs can mimic the effect of thyroid hormones [37, 84, 85] and that thyroid hormones can mimic the effect of PPs [84, 86]. Furthermore, thyroid hormones possess some hypolipidemic activity [14], and thyroid hyperplasia is occasionally observed in animals chronically administered phthalate esters [87]. Perhaps in some of these cases PPAR acts as a positive transcription factor, for example with some thyroid hormone responsive genes. In other instances, however, PPAR may antagonize the effects of thyroid hormones. Conceivably this could lead indirectly to the production of thyroid hyperplasia via the enhanced production of the goitrogen, thyroid stimulating hormone (TSH). It is possible that, depending upon the context of the gene promoter and the presence of ligand, receptors binding to the same response elements could either have the same or even opposite effects. As examples, RAR and TR may form heterodimers that are either synergistic or inhibitory depending upon the promoter [62], and the TR binds to an oestrogen response element and represses activation by the ER [61]. It is also possible that PPBP plays some role in modulating the effects of PP. For example, PPBP may have a similar role to play as the cellular retinoic acid binding proteins (CRABPs) which are thought to influence the level of retinoic acid available to bind to RARs [88].

It is possible that the carcinogenic effects of PPs are also mediated by PPAR. This could be due to oxidative damage to DNA resulting from elevated levels of hydrogen peroxide [17]. Alternatively, tumour formation could result from important changes in cellular growth and differentiation [89]. In this case PPAR may alter the expression of key genes relevant to growth and differentiation. Although such genes remain to be identified, it is possible that they include oncogenes [90, 91], growth factors or their receptors [92]. The identification of the mechanism by which not only PPs but also other non-genotoxic carcinogens such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), phenobarbital, phorbol esters and oestrogens influence tumourigenesis is an important area of research that should yield valuable insights into the mechanisms of chemical carcinogenesis.

*Species differences*

The phenomenon of peroxisome proliferation, including both the hyperplastic and hypertrophic response, has been examined in a number of species using either intact animals or cultured hepatocytes. Several markers of the response have been measured including peroxisome morphometrics and the induc-

tion of enzymes such as ACO and P450 IV [73, 93–98] and hyperplasia [22]. In each case, mouse and rat are the most responsive, hamster is intermediate, and guinea pig and monkey are either very weakly responsive or unresponsive. Importantly, no effects were seen in humans [99, 100] or when using human hepatocyte cultures [73]. Interestingly, in most species there is a good agreement between the induction of the hypertrophic (ACO, P450 IVA1) and hyperplastic markers (S-phase). For example, both are strongly induced in mice and rats with intermediate induction in hamsters and no induction in guinea pigs or humans [22, 73]. Generally this may indicate that both aspects of the peroxisome proliferation response are activated through a common mechanism. However, in the monkey no induction of ACO was observed in response to nafenopin even though there was a small stimulation of S phase in serum free conditions [101]. As yet, little is known about the susceptibility of each of these species to the carcinogenic action of PPs. Such studies are obviously important in attempting to define a link between PP action and cancer.

The basis for the species differences in response to PPs is unlikely to be due to differences in metabolism or pharmacokinetics since primary hepatocyte cultures prepared from these species also demonstrate a differential response [73, 97]. The variability therefore appears to reflect differences in the way that hepatocytes respond to the PP stimulus. If peroxisome proliferation is mediated by PPAR, then such species differences could reflect either variation in PPAR or in the gene networks that are regulated by PPAR. It will therefore be of interest to examine both the level of PPAR expression as well as the activity of PPAR in response to PP amongst species.

If PPAR is required to mediate the effects of PP, then determining the activity of PPAR amongst species and especially in humans would have important consequences in assessing the hazard PPs pose to humans.

*Conclusions*

Clearly the data so far support a model where PPAR is the mediator of PP action (Fig. 3). By analogy with the mechanism of action of other nuclear hormone receptors, PPAR would be activated by the binding of PP, would recognize specific DNA sequence motifs located upstream of PP target genes (PPRE), and would activate specific gene transcription. Such target genes would include those of the $\beta$-oxidation enzymes and P450 IVA1 but could also include genes important in the hyperplastic and carcinogenic response.

It will be important to determine whether all of the effects of PPs are receptor-mediated. If they are, then learning more about the role and function of PPAR presents an exciting and unique opportunity to understand more about the role of PPs in hypolipidaemia, peroxisome proliferation and cancer. Furthermore, examination of the expression and function of PPAR in humans could have important implications in assessing the hazard that PPs may represent to humans. Identification of the putative natural ligand combined with an increased knowledge of the structure and function of PPAR gained from molecular, biochemical and crystallographic analyses should help to improve drug and chemical design. It may be possible to improve chemical specificity by, for example, designing leukotriene antagonists, herbicides or plasticizers that are devoid of PP activity. In addition, a rational drug design approach could be employed to produce second generation hypolipidemic drugs that are both more potent and selective with which to treat chronic heart disease.

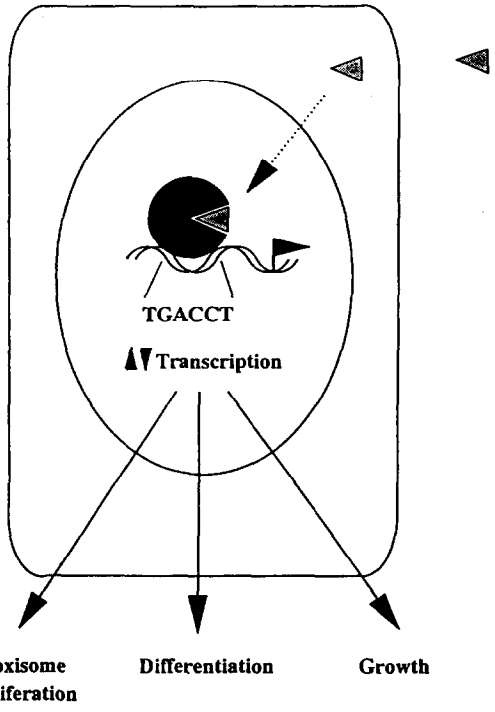


Fig. 3. Model of peroxisome proliferator action. Peroxisome proliferators are assumed to enter the cell by diffusion and either to bind directly to the receptor (PPAR) or to perturb lipid metabolism leading to induction of the proximate peroxisome proliferator. PPAR is shown located in the nucleus and is speculated to recognize TGACCT motifs located upstream of target genes. The activated receptor would then enhance or repress gene transcription resulting in peroxisome proliferation, changes in differentiation and growth. It is proposed that some of these events are relevant to the carcinogenic mechanism of peroxisome proliferator action.

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