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ON THE MECHANISM OF INDUCTION OF MICROSOMAL CYTOCHROME P450IVA1 AND PEROXISOME PROLIFERATION IN RAT LIVER BY CLOFIBRATE

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Abstract—The time course of induction of microsomal and peroxisomal lipid-metabolizing enzymes in male Wistar rat liver has been investigated following a single i.p. dose of clofibrate (250 mg/kg). The microsomal enzyme, cytochrome P450IVA1, demonstrated a biphasic response to sodium clofibrate administration, the biphasic response consisting of an initial small response, peaking at approximately 30 min post-dose and returning to near baseline values after 2 hr. A second major induction of cytochrome P450IVA1 occurred between 18 and 24 hr post-dose. This biphasic phenomenon for cytochrome P450IVA1 was observed for the enzyme activity (lauric acid hydroxylase), immunodetectable protein (using a specific ELISA method) and at the mRNA level (using a 2.1 kilobase cytochrome P450IVA1 cDNA probe). In contrast, peroxisomal fatty acid β-oxidation enzymes responded in a monophasic manner to clofibrate administration, peaking approximately 24 hr post-dose. Accordingly, microsomal cytochrome P450IVA1 was induced before the peroxisomal enzymes of fatty acid β-oxidation. The effect of cycloheximide on the induction of peroxisome proliferation by clofibrate was additionally investigated. The prior administration of cycloheximide to Wistar rats ablated the clofibrate-dependent induction of both cytochrome P450IVA1 and peroxisomal-dependent lipid metabolism and also blocked the corresponding synthesis of enzyme proteins. Cycloheximide additionally inhibited the clofibrate-dependent increase in peroxisomal acyl-CoA oxidase mRNA, but was without effect on the induced cytochrome P450IVA1 mRNA levels, indicating a protein or enzyme dependency for the phenomenon of peroxisome proliferation. Taken collectively, our data strongly argues that the regulation of microsomal cytochrome P450IVA1 and peroxisomal fatty acid β-oxidation enzymes are closely related, possibly through the initial, clofibrate-dependent regulation of cytochrome P450IVA1.

The administration of a wide range of xenobiotics to rats and mice results in a hepatomegaly which is characterized by a pronounced proliferation of cellular organelles, including peroxisomes and the smooth endoplasmic reticulum [1], and some of these compounds (termed peroxisome proliferators), have been associated with an increased incidence of hepatocellular carcinomas in the rat [2]. Although the precise relationship between the short term changes observed upon exposure of rats to xenobiotic peroxisome proliferators and tumour formation is uncertain, it has been suggested that hepatocarcinogenesis is related to the sustained proliferation of peroxisomes, although peroxisome proliferators *per se* are not genotoxic [2]. Recently, increased levels of oncogene products have been observed within 28 days of treatment with nafenopin and have been implicated in the process of tumour formation [3], although the precise details and molecular mechanisms of regulation remain to be established.

Concomitant with these xenobiotic-dependent subcellular changes, is an induction of several proteins and enzyme activities including the enzymes of peroxisomal β-oxidation, an isozyme of cytochrome P450 (termed cytochrome P450IVA1, responsible for the ω-hydroxylation of fatty acids), carnitine acetyl transferase, carnitine palmitoyl transferase, cytosolic epoxide hydrolase and fatty acid binding protein [1, 4]. These proteins are thought to form a "peroxisome proliferator domain" similar to that of the Ah locus [5]. Although these proteins appear to be co-induced, the precise temporal inter-relationship between them is unknown, as is their mechanism of induction. It has been suggested that the mechanism of peroxisome proliferation involves a soluble cytosolic receptor [6], or a peroxisome proliferator binding protein (PPbP) [7], although other workers have been unable to verify the existence of such a protein receptor [8, 9]. Recently it has been suggested that the induction phenomena are a response to perturbations in hepatocellular lipid metabolism and that the induction of the individual lipid metabolizing enzymes may be temporally distinct [10, 11].

Accordingly, the present study examines the temporal relationship and protein dependency of induction between several of the proteins and enzyme activities of the peroxisome proliferator domain in order to further examine the mechanism of the induction process.

MATERIALS AND METHODS

Chemicals. Sodium clofibrate was a gift from I.C.I. p.l.c., Pharmaceuticals Division (Macclesfield,

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U.K.). Lauric acid, benzphetamine, *t*-butylhydroperoxide and NADPH were purchased from the Sigma Chemical Co. (Poole, U.K.) and [1-^{14}C]lauric acid was supplied by the Radiochemical Centre (Amersham, U.K.) All other chemicals were obtained from commercial sources and were of the highest purity available.

Antibodies and cDNA probes. Electrophoretically homogeneous cytochrome P450IVA1 protein and anti-P450IVA1 serum were provided by Dr R. Sharma (University of Surrey), as previously described [10]. Anti-peroxisomal bifunctional protein serum was a kind gift from Dr D. Cinti (University of Connecticut Health Center, CT, U.S.A.). Pre-immune sera and enzyme labels were provided by Guildhay Antisera Ltd (Guildford, U.K.). A 2.1 kilobase cDNA probe to cytochrome P450IVA1 was provided by Dr C. McGeoch (University of Surrey) and cDNA probes to peroxisomal bifunctional protein and peroxisomal acyl-CoA oxidase were a kind gift of Dr T. Hashimoto (Matsumoto, Japan).

Animal and drug pretreatment. For the analysis of the time course of induction, male Wistar albino rats (150–200 g initial body wt, University of Surrey breeders) were injected i.p. with a single dose of either sodium clofibrate (250 mg/kg) or saline (0.9% w/v), and the animals killed at varying times after dosing, ranging from 15 min to 40 hr. The livers were subsequently removed, and 1 g portions were frozen immediately in liquid nitrogen and stored at −70° until required for RNA isolation. The livers were then perfused with 0.9% (w/v) saline prior to homogenization. Samples of whole liver homogenate were taken for the determination of peroxisomal palmitoyl CoA oxidation and bifunctional protein. Microsomal fractions were prepared by ultracentrifugation [10] for the determination of lauric acid hydroxylase activity and ELISA quantitation of cytochrome P450IVA1.

For the analysis of the effect of cycloheximide on the induction of peroxisomal proliferation by sodium clofibrate, male Wistar rats were injected i.p. with a combination of doses given 90 min apart. The dosing regimes were either sodium clofibrate (250 mg/kg) followed by cycloheximide (2 mg/kg); cycloheximide followed by sodium clofibrate; saline followed by sodium clofibrate; saline followed by cycloheximide or saline followed by saline. All animals were killed 24 hr after the initial dose. The livers were removed and processed as previously described.

Enzyme assays. Total cytochrome P450 was determined according to Omura and Sato [12] using a difference absorption coefficient (450–490 nm) of 91 $mM^{-1}\,cm^{-1}$ for the sodium dithionite-reduced carbon monoxide adduct. The clofibrate-induced cytochrome P450IVA1 enzyme and peroxisomal bifunctional protein were determined immunochemically by an ELISA method as described by Sharma *et al.* [10].

The 11- and 12-hydroxy metabolites of ^{14}C-labelled lauric acid [13] were separated by reverse-phase HPLC using a Micropak MCH-10 column (30 × 0.4 cm, Varian Associates Ltd, Walton-on-Thames, Surrey, U.K.). The metabolites were resolved using a linear gradient of water : methanol (45:55 containing 0.1% acetic acid) to 100% methanol over a 35 min period at a flow rate of 1.5 mL/min. The HPLC eluate, containing radioactive metabolites was passed through a Berthold LB503 Radiodetector flow-cell (Lab-Impex, Twickenham), interfaced with a Commodore PET (Series 4000), enabling quantitative analyses of lauric acid metabolism. Alternatively, total (11- + 12-hydroxy) lauric acid hydroxylase activity was determined by TLC as previously described [13] and quantitated using a Berthold TLC linear-analyser plate scanner (Berthold, U.K.).

Tissue whole homogenates were assayed for KCN-insensitive palmitoyl-CoA oxidation activity as described by Bronfman *et al.* [14] and protein concentration was determined by the method of Lowry *et al.* [15] using crystalline bovine serum albumin as the standard.

RNA extraction and cDNA hybridization. Total RNA was isolated by the methods of Chomczynski and Sacchi [16] from tissue that had been frozen and stored at −80°. RNA dot blot analysis was carried out using the method of White and Bancroft [17]. The filters were probed with ^{32}P-labelled cDNA probes, generated by nick translation [18] and hybridization conditions were as previously reported [19]. The hybridized filters were subjected to autoradiography with fast X-ray film (Kodak GBX2) in an X-ray cassette containing an intensifying screen, for 4 days at −80°. A semi-quantitative estimate of the intensity of the spots on the developed X-ray film was made using a Gelman densitometer.

RESULTS

The administration of a single i.p. dose of sodium clofibrate (250 mg/kg) results in changes in liver enzymology classically associated with the administration of peroxisome proliferators to rats. Significant changes in fatty acid metabolism were observed, namely increases in lauric acid 11- and 12-hydroxylase and cyanide-insensitive, palmitoyl-CoA oxidation (Fig. 1) These activities were increased to maximal levels of 615 and 378%, respectively. The time courses of induction of these two enzyme activities however were different from each other. Lauric acid hydroxylase, the marker activity for cytochrome P450IVA1, was induced in a biphasic manner with peaks of activity occurring approximately 30 min and 18 hr post-dose. These time-dependent, biphasic increases in lauric acid hydroxylase activities were mirrored by a biphasic increase in cytochrome P450IVA1 apoprotein content (determined by ELISA) after inducer pretreatment (Fig. 1). In contrast, the peroxisomal β-oxidation activity, as measured by cyanide insensitive palmitoyl-CoA oxidation, did not show such a biphasic response, but rather a single peak of activity occurring approximately between 18 and 24 hr post-dose.

Total RNA extracted from groups of rats after various times post-dose was subjected to dot blot analysis using cDNA probes to cytochrome P450IVA1 and acyl-CoA oxidase. Again, the cytochrome P450IVA1 mRNA level exhibited a biphasic response, peaking at approximately 30–

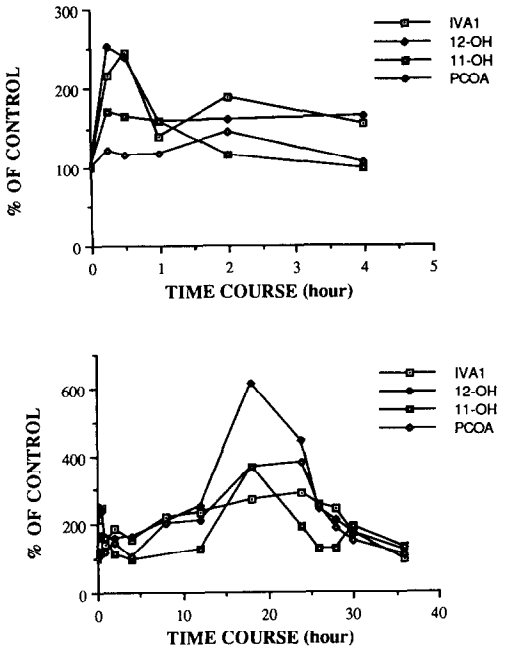

Fig. 1. Time course of induction of hepatic lipid-metabolizing enzymes. Groups of rats were administered a single i.p. injection of clofibrate, liver fractions isolated at various time points and analysed for cytochrome P450IVA1 apoprotein (IVA1), lauric acid hydroxylase (12-OH and 11-OH) and palmitoyl CoA-oxidation (PCoA) activities, determined as described in Materials and Methods. Each time point is the mean of duplicate determinations of three samples (zero time point is the mean of duplicate determinations of eight samples), and statistical significance is omitted for clarity. 100% Control values (time 0) correspond to: 1.59 ± 0.52 nmol 11-hydroxy lauric acid formed/min/nmol P450; 2.74 ± 1.35 nmol 12-hydroxy lauric acid formed/min/nmol P450; 5.29 ± 1.33 nmol NADH formed/min/mg protein. The 100% value for P450IVA1 ELISA determination was 1.75 ± 0.74% of the total, carbon monoxide-discernible cytochrome P450.

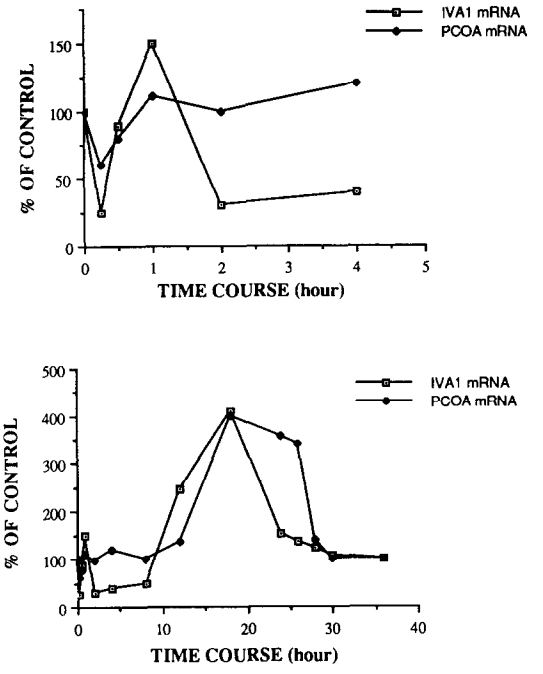

Fig. 2. Temporal induction of cytochrome P450IVA1 and acyl CoA-oxidase mRNAs by clofibrate in rat liver. Total hepatic RNA was isolated from groups of rats various times after clofibrate administration and blotted against cytochrome P450IVA1 (IVA1) and acyl CoA-oxidase (PCoA) cDNA probes. The dot blots were scanned by densitometry and the control values (zero time point) assigned an arbitrary value of 100%. The values represented are the average of duplicate determinations, each time point consisting of 5 μg of pooled RNA isolated from three individual animals.

60 min and 18 hr post-dose, whereas the acyl CoA-oxidase mRNA levels exhibited a monophasic response to the inducer, peaking at 18 hr (Fig. 2). The mRNA levels coding for both these proteins exhibited a small drop at the earliest time point studied (15 min), probably as a result of the injection trauma. These overall changes in mRNA levels were a true reflection of inducer-dependent changes as actin mRNA levels and sham-injected animals demonstrated no response (data not shown).

The effect of cycloheximide treatment on the induction of some of the proteins of the peroxisome proliferator domain was studied in order to further elucidate the mechanism of induction. Cycloheximide administration prior to the administration of a single i.p. dose of sodium clofibrate resulted in the total abolition of the inductive effect of the sodium clofibrate in every dose group. This was seen at the level of total (11- + 12-) lauric acid hydroxylase activity and cytochrome P450IVA1 apoprotein content (Fig. 3A) and peroxisomal β-oxidation activity and bifunctional apoprotein content (Fig. 3B). The influence of cycloheximide on both cytochrome P450IVA1 and acyl CoA-oxidase mRNA levels were also examined. As shown in Fig. 4, cycloheximide treatment did not significantly influence the clofibrate-dependent induction of cytochrome P450IVA1 mRNA, but totally blocked the induction of the peroxisomal acyl CoA-oxidase mRNA, indicating a protein-dependency for the induction of the peroxisomal enzyme. Quite clearly, the above cycloheximide-mediated blockade of clofibrate-induced acyl CoA-oxidase mRNA cannot simply be explained by a non-specific toxicity of cycloheximide, as the P450IVA1 mRNA levels are still elevated under the same experimental conditions (Fig. 4).

The administration of sodium clofibrate prior to that of cycloheximide was found, surprisingly, to be lethal to five out of six rats with deaths occurring between 4 and 24 hr post-dose, and therefore no data is shown for this particular dose group. The reason for this observation is at present unknown.

DISCUSSION

The results of this study demonstrate that the

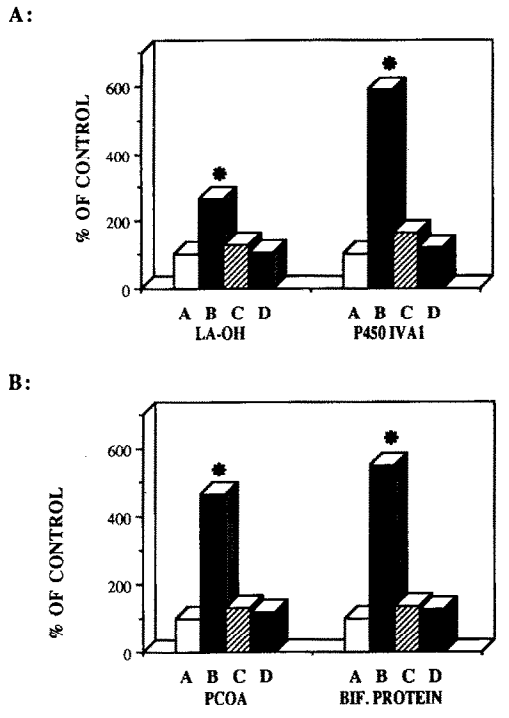

Fig. 3. Influence of cycloheximide on the clofibrate-dependent induction of hepatic lipid-metabolizing enzymes in the rat. Groups of three rats were treated with either saline (0.9%), clofibrate (250 mg/kg) or cycloheximide (2 mg/kg), dosed 90 min apart, and killed 24 hr after the initial dose as follows: Group A, saline plus saline; Group B, saline plus clofibrate; Group C, cycloheximide plus clofibrate; Group D, cycloheximide plus saline. (A) Microsomal enzymes. Total (11- + 12-) hydroxy lauric acid (LA-OH) formed (100% control value, saline plus saline: 2.17 ± 0.30 nmol products formed/min/nmol total P450) and specific cytochrome P450IVA1 apoprotein (100% control value, saline plus saline: 8.66 ± 1.83 pmol P450IVA1/mg microsomal protein. (B) Peroxisomal enzymes. Peroxisomal palmitoyl CoA-oxidation (PCoA) (100% control value, saline plus saline: 4.35 ± 0.57 nmol NADH formed/min/mg and bifunctional (BIF) protein content (100% control value, saline plus saline: ng control protein equivalents/μg total protein). Data are expressed as the mean from three individual animals and are significantly different from the control at $P < 0.001$ (Student's *t*-test) where indicated (★).

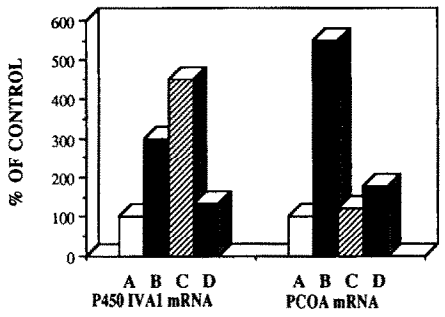

Fig. 4. Influence of cycloheximide on the clofibrate-dependent induction of cytochrome P450IVA1 and acyl CoA-oxidase mRNAs in rat liver. Groups of three rats were treated as described in the legend to Fig. 3, total RNA extracted and hybridized with cDNA probes to cytochrome P450IVA1 and acyl CoA-oxidase as described in Materials and Methods. The dot blots were scanned by densitometry and an arbitrary value of 100% assigned to the control groups (saline plus saline treatment).

mechanism of induction of cytochrome P450IVA1 and peroxisomal β-oxidation by sodium clofibrate are related but yet temporally distinct phenomena.

The proteins of the peroxisome proliferator domain are induced by a vast range of structurally diverse chemicals, including hypolipidaemic drugs such as clofibrate and its analogues; plasticisers, e.g. DEHP; pesticides such as dimethrin and 2,4,5-T; drugs, such as aspirin and valproic acid; and industrial solvents such as isooctane and trichloroethylene [1, 4]. It has been hypothesized that the mechanism of induction of peroxisome proliferation is mediated by a soluble cytosolic receptor [6, 7]. In order to account for the vast structural diversity of the xenobiotic inducers it has been speculated that such a receptor either has more than one ligand binding site or exists in more than one subtype. Saturable displaceable specific binding has been detected in rat liver cytosol by Lalwani *et al.* [6], and the putative receptor has been purified [7]. However, other workers have been unable to reproduce this data [8, 9]. This has led to the proposal of an alternative mechanism of induction [10, 11]. These latter workers have invoked a substrate overload mechanism of induction and it has been proposed that the induction of peroxisomal β-oxidation is dependent on the prior induction of cytochrome P450IVA1 [10]. The data in this paper is not inconsistent with such a sequential mechanism, and it appears that the induction of lauric acid hydroxylase activity occurs before that of peroxisomal β-oxidation.

The observation that the P450IVA1 mRNA peaks after the apoprotein in the short term (Figs 1 and 2), may reflect the relative specificities of the antibody and cDNA probes. The P450IVA1 cDNA:RNA hybridizations were performed under conditions of high stringency and it is likely that only the P450IVA1 mRNA was detected. In contrast, the antibody used to quantitate the P450IVA1 apoprotein also recognises an additional band of lower molecular weight, which presumably is another closely-related member of the P450IVA1 sub-family [20]. Accordingly, it is possible that this lower molecular weight orthologue is preferentially induced at an earlier time point than P450IVA1, thus "skewing" the apoprotein peak in the time course.

The biphasic response of cytochrome P450IVA1 is intriguing. This phenomenon is extremely rapid and has not, to our knowledge, been previously observed. To rationalize this observation, it is necessary to further analyse the proposed sequential mechanism of induction. In this scheme [10], the initial stimulus responsible for the induction of cytochrome P450IVA1 has not been clearly defined. However, it has been proposed that the initial stimulus is a perturbation in the normal fatty acid metabolism of the hepatocyte in response to mitochondrial enzyme inhibition by the xenobiotic.

It has been shown that a metabolite of MEHP, namely metabolite VI or 5-keto MEHP, inhibits [^{14}C]palmitate oxidation in isolated rat hepatocytes [21]. Several other xenobiotic peroxisome proliferators have also been shown to inhibit enzymes of mitochondrial fatty acid metabolism in the hepatocyte [22–25]. Many of these compounds inhibit carnitine palmitoyltransferase I, which functions in the transport of fatty acid CoA-esters into the mitochondria. Any such interference with hepatocellular fatty acid metabolism will likely result in an accumulation of intracellular fatty acids. Indeed, that transient accumulation of lipid following the administration of peroxisome proliferators is a well documented phenomenon [24]. The nature of this accumulated lipid is unknown, but it has been hypothesized that the lipid may consist of free fatty acids or their CoA esters [11].

This lipid overload hypothesis can also accommodate the induction of peroxisome proliferation by non-xenobiotic inducers. Such inducers include starvation, diabetes, cold adaptation and high fat diets [1, 4]. All these latter manipulations result in an increased flux of fatty acids through the hepatocyte, perturbing normal hepatocellular fatty acid homeostasis. The biphasic response in cytochrome P450IVA1 may reflect subtle differences in the levels or composition of accumulated fatty acids and the hepatocyte's ability to deal with this perturbation. It may not be surprising to find that the hepatocyte responds rapidly to any changes in hepatic fatty acid homeostasis as it is well documented that certain fatty acids, such as polyunsaturated fatty acids and long chain dicarboxylic fatty acids, cause mitochondrial swelling and loss of function [26, 27]. It is interesting to note that these compounds are metabolized by the peroxisome [28, 29], reflecting the important role of the peroxisome in hepatocellular fatty acid metabolism.

The exact mechanism by which the fatty acid build up causes increased gene transcription is, as yet, unclear. This may function by way of specific DNA binding protein(s). Analysis of the 5′-regulatory regions of the genes of the peroxisome proliferator domain may provide insight into the *cis*-acting factors involved. Interestingly, it has been shown that genes for several of the enzymes of the peroxisome proliferator domain contain GC-rich regions [30–32], but whether or not this is related to the phenomenon of proliferation remains to be investigated.

Our investigation of the effect of inhibition of protein synthesis by cycloheximide produced results that were not inconsistent with the hypothesis that the induction of peroxisomal β-oxidation requires the prior induction of cytochrome P450IVA1. Interestingly, the administration of sodium clofibrate prior to that of cycloheximide was toxic to five out of the six animals. This may be interpreted as meaning that the initial administration of sodium clofibrate generates a toxic stimulus (possibly a build up of fatty acids) which requires *de novo* protein synthesis to be overcome. The reverse dosing protocol (i.e. cycloheximide followed by sodium clofibrate) does not generate such a toxic insult. Whether this lethality is related to the biphasic response described herein is unknown. Furthermore, it would be extremely informative to determine whether this is a compound specific phenomenon and to study the effect of these dosing protocols in non-responsive species such as the guinea-pig.

In conclusion, the data presented in this paper further substantiates the proposed lipid overload theory for the induction of peroxisome proliferation by xenobiotics, although the precise molecular aspects of cytochrome P450IVA1 induction and associated peroxisome proliferation remain to be established.

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