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Localization and differential induction of cytochrome *P*450IVA and acyl-CoA oxidase in rat liver

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The peroxisome proliferators are structurally diverse chemicals which induce hyperplasia, hypertrophy and the proliferation of peroxisomes in the rodent liver. Cytochrome *P*450IVA1 and peroxisomal enzymes, such as acyl-CoA oxidase, are induced and are early markers of treatment with peroxisome proliferators. In this study, rats were dosed intraperitoneally with the potent peroxisome proliferator methylclofenapate and the hepatic induction response was studied. There was no significant change in the enzyme activities of laurate hydroxylase (cytochrome *P*450IVA1) or acyl-CoA oxidase in the first 8 h after treatment, but the activities had doubled at 24 h, suggesting that these enzymes are not involved in the mediation of early events in peroxisome proliferation. Hepatic cytochrome *P*450IVA1 mRNA was significantly increased at 6 and 8 h after treatment, rising to 15-fold above control values at 30 h. In contrast, acyl-CoA oxidase mRNA showed no significant change in the first 8 h, but increased to 13-fold above control values at 24 and 30 h, thereby demonstrating different kinetics of induction of the two mRNAs. In order to determine whether cytochrome *P*450IVA1 and peroxisomal enzymes were included in the same cells, rats were treated daily with sub-maximal (2 or 5 mg/kg) and maximal (25 mg/kg) inducing doses of methylclofenapate for 4 days. The lobular distribution of induced proteins was determined immunocytochemically with antibodies raised against *P*450IVA1 and acyl-CoA oxidase. Livers from control animals showed minimal staining for both proteins. However, in the livers of animals treated with 2 or 5 mg of methylclofenapate/kg, both acyl-CoA and *P*450IVA immunostaining was increased, mainly in the centrilobular area. Immunostaining of serial sections revealed that these proteins were induced in the same region of the lobule. A maximal inducing dose of methylclofenapate (25 mg/kg) caused panlobular induction of both proteins. The results demonstrate that these proteins are induced in a dose-dependent manner in the same, spatially distinct, sensitive region of the liver lobule.

INTRODUCTION

The peroxisome proliferators are a structurally diverse class of chemicals which increase the number and volume of peroxisomes in hepatocytes [1,2], and also the number of these cells undergoing DNA synthesis after acute exposure [3]. These changes are associated with the induction of several proteins, notably a microsomal cytochrome *P*-450 which hydroxylates fatty acids [4,5], and enzymes involved in peroxisomal fatty acid β-oxidation pathways, such as acyl-CoA oxidase (EC 1.3.3.6; ACO). The mechanism whereby these changes occur in hepatocytes is currently unknown, but two hypotheses have been proposed. These are (*a*) that there is a specific receptor for peroxisome proliferators (or their metabolites) which mediates gene activation [6–8], and (*b*) that substrate overload occurs, a consequence of peroxisome-proliferator-mediated perturbation of lipid metabolism, which leads to changes in gene activation [2].

The ability of structurally diverse chemicals, as well as 'physiological' stimuli such as high-fat diets and diabetes [8,9], to induce peroxisome proliferation led to rationalization of the observation that the *P*-450 and peroxisomal enzymes which are induced are involved in lipid metabolism. Lock *et al.* have proposed a mechanism whereby peroxisome proliferation is an adaptive response to perturbation of lipid metabolism [2]. They propose that cytochrome *P*450IVA1 is induced by medium-chain fatty acids accumulation, and that *P*450IVA1 hydroxylates fatty acids to give dicarboxylic acids [11,12], which are known to induce acyl-CoA oxidase [2]. This proposal requires that cytochrome *P*450IVA1 be induced before the induction of peroxisomal enzymes.

A cDNA encoding cytochrome *P*450IVA1, cloned from rat liver [11,13], has been shown to catalyse the ω-hydroxylation of lauric acid [11] and arachidonic acid [12] and is inducible by clofibrate [14]. However, the kinetics of induction of peroxisomal enzymes are currently confused, as several groups have studied the induction of peroxisomal enzyme mRNAs with different results [14–16]. The comparative induction kinetics of cytochrome *P*450IVA1 and peroxisomal enzyme RNAs have not been examined to date.

There is evidence *in vitro* of hepatocyte populations which are differentially sensitive to induction of cytochromes *P*-450 by the peroxisome proliferator clofibric acid [17]. In addition, Styles *et al.* [3] demonstrated the presence of a sub-population of hepatocytes which are differentially sensitive to the mitogenic effects of peroxisome proliferators. These results suggest that liver cell population(s) may exhibit heterogeneity in the induction of either microsomal or peroxisomal enzymes by peroxisome proliferators. The lipid overload mechanism of peroxisome proliferation [2] would require that the microsomal and peroxisomal enzymes would be induced in the same cell populations.

We examined the hepatic induction of microsomal cytochrome *P*450IVA1 and peroxisomal ACO enzymes and their corresponding mRNAs after a single dose of the peroxisome proliferator methylclofenapate. The high sensitivity and specificity of the RNAase protection assay enabled the demonstration of differing induction kinetics of the *P*450IVA1 and ACO mRNAs. Further, in order to investigate whether cytochrome *P*450IVA1 and ACO are induced in the same cells, we used immunocytochemical localization within the hepatic lobules of methylclofenapate-treated rats to demonstrate that the peroxisomal and

Abbreviations used: ACO, acyl-CoA oxidase; LAH, lauric acid hydroxylase.

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microsomal enzymes show a dose-dependent induction within the lobular architecture, and that both proteins are localized in the same sensitive sub-population of hepatocytes.

MATERIALS AND METHODS

Materials

Peroxidase-labelled goat anti-rabbit and rabbit anti-sheep IgG were obtained from ICN Biomedicals, Buckingham, U.K. and DAKO Ltd., High Wycombe, Bucks., U.K. respectively. 3,3'-Diaminobenzidine was obtained from Polysciences, Northampton, U.K. [α-^{32}P]CTP (800 Ci/mmol) was from Amersham International, Amersham, Bucks., U.K. All other chemicals were of the highest purity available.

Male Alderley Park rats (180–200 g) were dosed intraperitoneally with methylclofenapate (synthesized by Lancaster Synthesis Ltd., Eastgate, WhiteLund, Morecambe, Lancs., U.K.), at 25 mg/kg body wt. and groups of three rats were killed by cervical dislocation at 0, 0.5, 1, 2, 4, 6, 8, 24 or 30 h after dosing. Control animals were dosed with vehicle alone (corn oil). Rats were starved overnight before killing. Determination of ACO and laurate hydroxylase (LAH) activities and RNA levels was performed on each liver sample.

For immunocytochemical studies, groups of two rats were administered methylclofenapate (2, 5 or 25 mg/kg body wt.) by gavage in 5 ml of corn oil/kg body wt. for each of 4 consecutive days and were killed by an overdose of halothane 24 h after the last dose. Controls were administered vehicle alone.

Isolation of RNA

Livers were frozen in liquid N_2 immediately after dissection and stored at -80 °C. RNA was isolated by lithium chloride precipitation from guanidinium thiocyanate [18]. RNAs were normalized by hybridization to oligo(dT)$_{18}$ and quantified by liquid scintillation counting [19].

Construction of plasmids

Plasmid pMJ115, encoding a partial cDNA of rat acyl-CoA oxidase [20], was kindly given by Dr. T. Osumi (Shinshu University, Shinshu, Japan). A 227 bp *Hin*dIII fragment, from the 3' non-coding region of the cDNA (residues 2050–2277), was subcloned into pGEM7 (Promega) to give the plasmid pACO.227. The plasmid was linearized with *Bam*HI to give a template for transcription with T7 polymerase. A full-length rat *P*450IVA1 cDNA was cloned into pUC9 as described by Earnshaw *et al.* [13]. A 1192 bp cDNA fragment (residues 965–2157) was excised by double digestion with *Taq*I and *Eco*RI and subcloned into pGEM7 digested with *Eco*RI and *Cla*I to give plasmid pIV2. This construct was digested with *Alu*I for transcription of 515 bp transcript with T7 polymerase.

RNAase protection analysis

Protection analysis was based on [21]. Linearized templates were treated with proteinase K, extracted with phenol/$CHCl_3$ (1:1, v/v) and ethanol-precipitated before transcription. RNA was synthesized in a buffer containing 40 mM-Tris/HCl (pH 7.5), 6 mM-$MgCl_2$, 2 mM-spermidine, 10 mM-NaCl, 10 mM-dithiothreitol (DTT), 1 unit of RNAasin/μl, 0.5 mM each of ATP, GTP and UTP, 1 μg of template DNA, [α-^{32}P]CTP and 10 units of T7 polymerase at 37 °C for 1 h. DNAase I was added and incubated for 15 min at 37 °C, followed by phenol/chloroform extraction and ethanol precipitation of the probe. Probe RNA was dissolved in deionized formamide and added to 30 μg of RNA in 80% formamide/40 mM-Pipes (pH 6.7)/0.4 M-NaCl/1 mM-EDTA in a volume of 30 μl. The hybridization mix was heated at 85 °C for 3 min and incubated at 37 °C overnight. Portions of 350 μl of 0.3 M-NaCl/10 mM-Tris/HCl/5 mM-EDTA (pH 7.5) containing 40 μg of RNAase A/ml and 2 μg of RNAase T1/ml were added to the hybridization mix and incubated at 37 °C for 1 h. Then 20 μl of 10% (w/v) SDS containing 50 μg of proteinase K was added and incubated at 50 °C for 15 min. The RNA was deproteinized by phenol/chloroform extraction and ethanol-precipitated. The precipitate was washed with 80% (v/v) ethanol, dissolved in 90% formamide/1 × TBE buffer [22] containing xylene cyanole, and electrophoresed on a denaturing 6% (w/v) acrylamide gel [22]. Gels were dried and autoradiographed, and band intensity was analysed using an LKB laser densitometer.

Northern blot analysis

RNAs were glyoxalated, electrophoresed and electroblotted on to Genescreen Plus according to standard protocols [22]. Hybridization to an ACO RNA transcript from pACO.227 was performed as described by Melton *et al.* [21], followed by washes at 60 °C in 0.1 × SSPE/0.1% SDS for 30 min. Oligonucleotides were constructed complementary to bases 1–39 of the sequence of *P*450IVA1 [11]: 5'-GATGGCTGCACCATGAGCGTCTCT-3' and 5'-GGTGGAGCTCAGTGCAGAGACGCTCAT-3'. These were annealed, extended with Klenow polymerase and hybridized according to [23]. Blots were washed at 37 °C in 0.1 × SSPE/0.1% SDS for 30 min.

Enzyme activity

Cyanide-insensitive fatty acid β-oxidation was measured as the palmitoyl-CoA-dependent reduction of NAD^+ in the presence of cyanide as described [24]. The enzymic activity was expressed as nmol of NAD^+ reduced/min per mg of protein. LAH activity (*P*450IVA1-dependent) was assayed using a t.l.c. procedure, as previously described [7,24]. LAH activity was expressed as nmol of hydroxylated lauric acid formed/min per mg of protein. Protein assay was by the Lowry method [25].

Statistics

Statistical analysis was performed using Student's t test, relative to concurrent controls; $P < 0.05$ was deemed to be significant.

Immunocytochemistry

Polyclonal antibodies to rat *P*450IVA1 were raised in sheep and prepared as described previously [12]. Rabbit polyclonal antibodies to rat ACO were a gift from Dr. T. Osumi [15,20]. Immunocytochemistry was performed as described [26,27]. After excision, the livers were sectioned into blocks of 2–3 mm thickness and immersed in 95% ethanol/1% acetic acid for 3 h at room temperature. Fixed tissues were embedded in paraffin and sections of 5 μm thickness were then mounted on to acetone-cleaned glass slides. Endogenous peroxidase was inhibited by treatment of sections with 5% H_2O_2 in 95% methanol at -20 °C for 30 min. After blocking non-specific binding sites, the sections were incubated with primary antibodies at dilutions ranging from 1:100 to 1:1000. Sections were counterstained in Harris' haemotoxylin, dehydrated and mounted in DPX (Dystrene Plasticizer in Xylene; BDH). Control incubations were performed by omission and by substitution of the first antibody with non-immune rabbit or sheep serum.

RESULTS

Northern blot analysis of ACO and *P*450IVA1 mRNA

RNA from the livers of rats treated with corn oil or with 25 mg of methylclofenapate/kg for 2 days was analysed by Northern

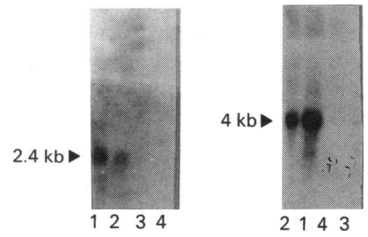

Fig. 1. Northern blot analysis of (*a*) *P*450IVA1 and (*b*) ACO transcripts in rat liver

Liver RNA (5 or 10 μg) from corn-oil- (lanes 3 and 4) or methylclofenapate (lanes 1 and 2) -treated rats was Northern blotted and hybridized to *P*450IVA1 oligonucleotides (*a*) or ACO cRNA (*b*), as described in the Materials and methods section. Size markers were ribosomal RNA bands (not shown). RNA used: lanes 1 and 3, 10 μg; lanes 2 and 4, 5 μg.

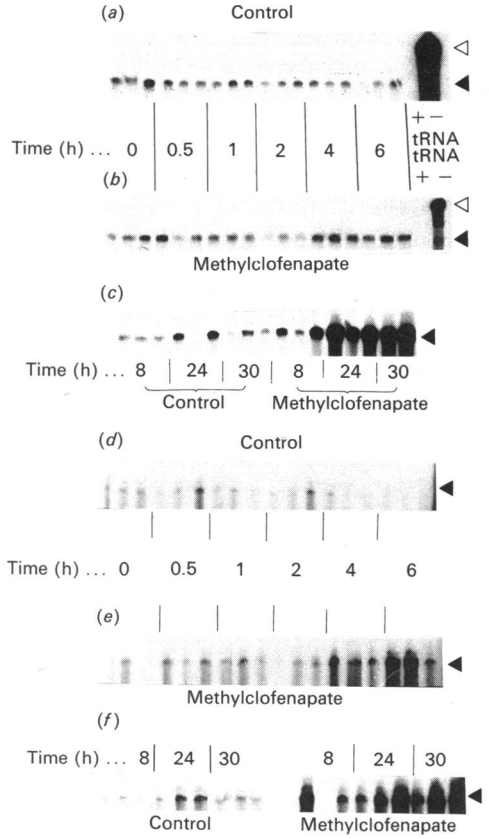

Fig. 2. Kinetics of induction of (*a–c*) ACO and (*d–f*) *P*450IVA1 mRNAs: RNAase protection

Liver RNA was isolated from each of three rats treated with corn oil or methylclofenapate at 0, 0.5, 1, 2, 4, 6, 8, 24 and 30 h after dosing. A 30 μg sample of RNA was analysed by a RNAase protection assay, as described in the Materials and methods section. The protected fragment is indicated by ◀ (227 bp for ACO; 515 bp for *P*450IVA1), and ◁ represents undigested probe. 'tRNA ±' represents cRNA probe incubated with yeast tRNA with or without RNAase treatment.

blot hybridization. An oligonucleotide probe complementary to bases 1–39 of the nucleotide sequence of *P*450IVA1 [11] was hybridized to a Northern blot of control and treated rat liver

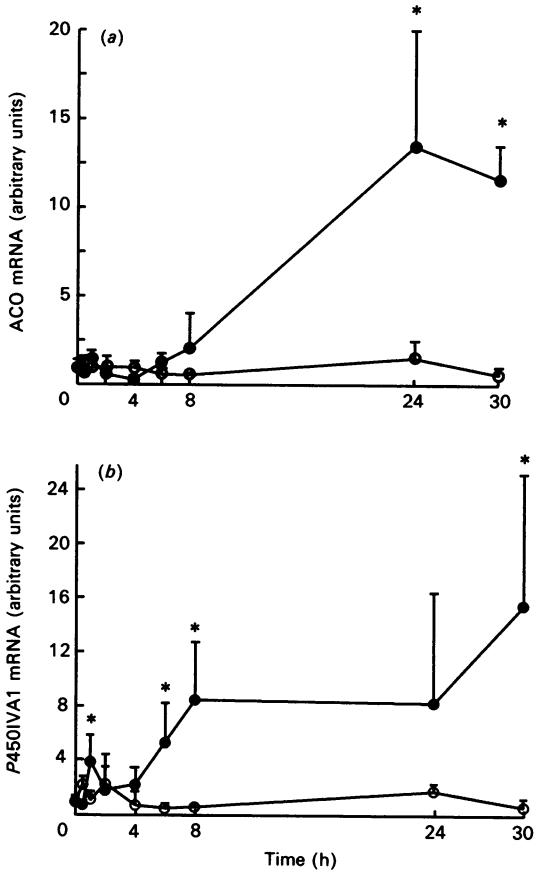

Fig. 3. Kinetics of induction of (*a*) ACO and (*b*) *P*450IVA1 mRNAs

RNAase protection analysis was performed on 30 μg of liver RNA from tissue isolated at various times after treatment of rats with corn oil (○) or methylcyclofenapate (●), as shown in Figs. 2(*a*)–2(*f*). RNAase protection analysis was on RNA from each of three animals per group and autoradiograms were analysed using an LKB laser densitometer. Values are plotted as means ± S.D., relative to time zero, which has an arbitrary value of 1. * Different from concurrent control, $P < 0.05$.

(Fig. 1*a*). A 2.4 kb message was present in RNA from rat liver treated with methylclofenapate, but was not detected in the livers of control animals, even after extended autoradiography.

Hybridization to an RNA transcript from pACO.227 revealed that an RNA of 4 kb was highly induced in the liver RNA of methylclofenapate-treated rats (Fig. 1*b*). The 4 kb mRNA species was present in control liver, but at a much lower concentration. Southern hybridization analysis of rat liver DNA with this probe revealed a single band (results not shown), confirming that the mRNA detected by Northern analysis is the product of a single gene [28].

Time course of induction of ACO and *P*450IVA1 mRNAs

The construction of pACO.227, containing 3′ non-coding sequences from the ACO cDNA, is described in the Materials and methods section. RNA transcripts were full length, and no background of protected bands was observed in yeast tRNA samples after RNAase treatment (Fig. 2). Fig. 2 shows a typical result from duplicate experiments analysing the time course of induction of ACO and *P*450IVA1 mRNAs after administration of a single intraperitoneal dose of methylclofenapate (25 mg/kg). These data were analysed by laser densitometry and the results are presented graphically in Fig. 3(*a*). The ACO mRNA content

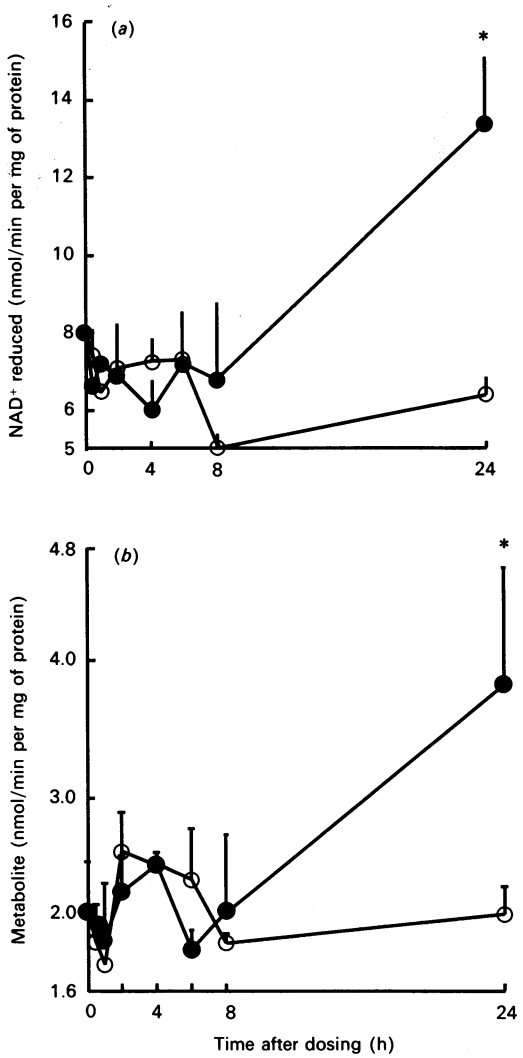

Fig. 4. Kinetics of induction of (*a*) cyanide-insensitive palmitoyl-CoA oxidation and (*b*) LAH after methylclofenapate treatment

Livers were taken from animals at the indicated times after treatment with corn oil (○) or methylclofenapate (●). Hepatic cyanide-insensitive palmitoyl-CoA oxidation (*a*) and LAH (*b*) were determined (as described in the Materials and methods section) from three rats per group. Values are expressed as means ± S.D. * Different from concurrent controls, $P < 0.05$.

in rats treated with corn oil varied by less than ± 25 % throughout the time period examined. However, the ACO mRNA content had increased by 13-fold at 24 h after treatment with methylclofenapate, and remained at a similar level 30 h after treatment (Figs. 2*a*–2*c* and 3*a*). No evidence was found for induction of ACO mRNA during the first 8 h after treatment with methylclofenapate.

A *P*450IVA1 cDNA clone [13] was subcloned as described in the Materials and methods section and pIV2 was digested with *Alu*I to give a transcript containing 515 bp of *P*450IVA1 3′ non-coding sequence. An example of results from duplicate experiment is shown in Figs. 2(*d*)–2(*f*), and these are displayed graphically in Fig. 3(*b*). When rats were dosed with methylclofenapate, the level of *P*450IVA1 mRNA had increased transiently (4-fold) at 1 h, but had returned to basal levels at 2 h after dosing. The *P*450IVA1 mRNA concentration increased significantly (by 5.5-fold) at 6 h after dosing, continuing to rise to 15-fold above control values at 30 h after treatment. Thus the increase in *P*450IVA1 mRNA occurred at least 2 h before any increase in ACO mRNA concentration.

Time course of enzyme activity induction

Enzyme activities for ACO and *P*450IVA1 were measured in rat livers at various time points after a single intraperitoneal dose of methylclofenapate. Cyanide-insensitive palmitoyl-CoA oxidation was determined in liver homogenates as a measure of peroxisomal ACO (Fig. 4*a*). No change was observed in the 8 h after dosing with methylclofenapate, but the activity was increased to 2-fold above control values at 24 and 30 h after dosing.

LAH was determined as a measure of *P*450IVA1 (Fig. 4*b*). LAH in control animals did not change appreciably throughout the time course, nor in the 8 h after dosing in methylclofenapate-treated animals. However, the enzyme activity increased 2-fold in treated animals at 24 and 30 h after dosing.

Immunocytochemistry

The antibody raised to *P*450IVA1 recognized two bands of similar apparent molecular mass in liver homogenates (results not shown), which are believed to be highly related members of the cytochrome *P*450IVA subfamily [11,29]; cross-reacting antigen is therefore designated as cytochrome *P*450IVA. The antibody raised against ACO has been described previously [15]. Liver sections from control animals were incubated with antibodies raised against *P*450IVA1 (Fig. 5*a*) or ACO (Fig. 5*d*), and showed slight immunostaining over the liver lobule, although staining for both was slightly stronger in the centrilobular region. When animals were given methylclofenapate at 2 or 5 mg/kg body wt. a clear pattern of lobular immunostaining was evident; intense staining for both *P*450IVA (Fig. 5*b*) and ACO (Fig. 5*e*) was restricted mainly to the centrilobular region. Moreover, immunocytochemical localization of *P*450IVA and ACO in consecutive serial sections revealed a strict geometric concordance between the lobular induction of the two enzymes. However, when animals were given 25 mg of methylclofenapate/kg body wt., immunocytochemistry revealed strong and homogeneous staining for both *P*450IVA (Fig. 5*c*) and ACO (Fig. 5*f*) throughout the liver lobule.

DISCUSSION

Analysis of the mechanism and kinetics of the response to peroxisome proliferators may elucidate many of the fundamental questions about the mechanism(s) of peroxisomal proliferation in the rodent liver. However, data describing the time course and magnitude of induction of microsomal and peroxisomal enzymes and their mRNAs is contradictory [2,11,14–16].

We have examined the activities of cyanide-insensitive palmitoyl-CoA oxidation (ACO) and LAH (*P*450IVA1) enzyme activities to determine if these enzymes were activated at an early stage following treatment with peroxisome proliferators. Both ACO and *P*450IVA1 enzyme activities were unchanged during the first 8 h after treatment, but rose to 2-fold above control values after 24 and 30 h (Fig. 4). Although LAH may not be completely specific for *P*450IVA1, the data presented suggest that it is unlikely that these enzymes have a mechanistic role in the mediation of the early response to peroxisome proliferator treatment, as described by Lock *et al.* [2]. It is, however, possible that these enzymes may mediate later events during peroxisome proliferation, such as the production of active oxygen or perturbation of lipid metabolism [2]. The slow accumulation of functional enzyme (2-fold at 24 and 30 h) may reflect a re-

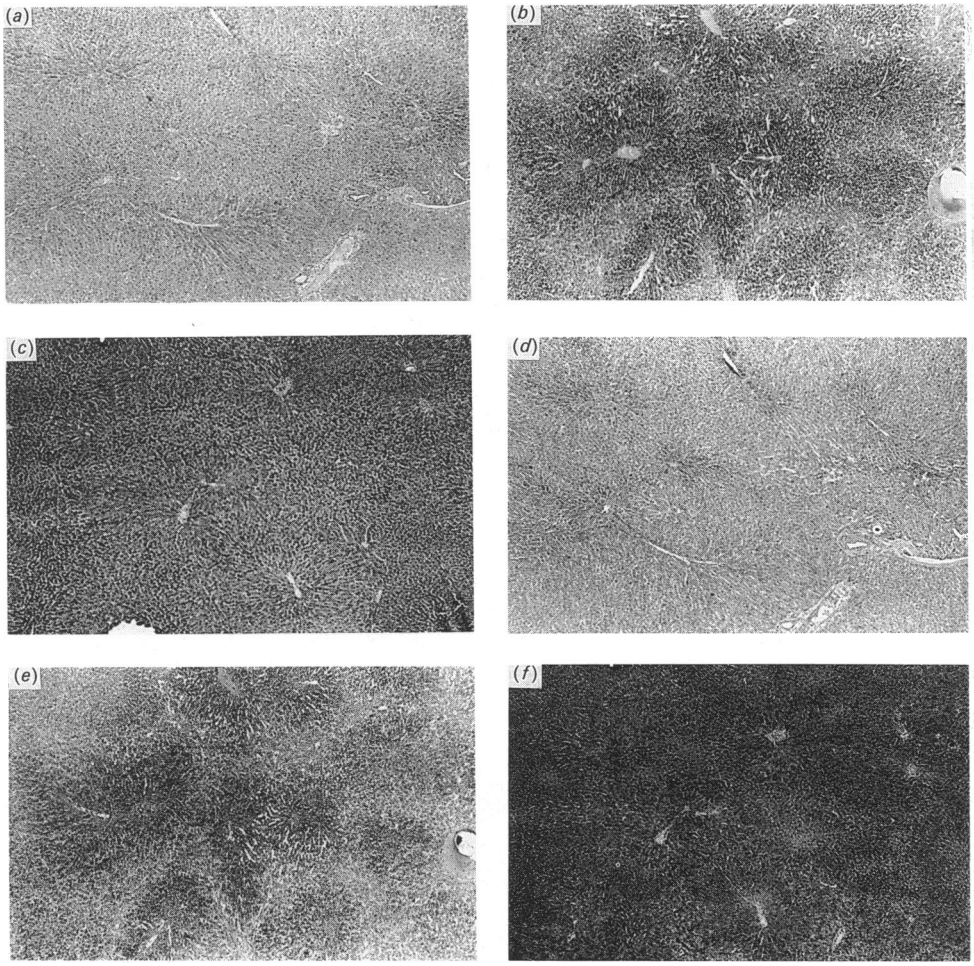

Fig. 5. Immmunocytochemical localization of (*a–c*) *P*450IVA1 and (*d–f*) ACO in the liver after treatment with methylclofenapate

Rats were treated daily with vehicle (*a*, *d*) or methylclofenapate [2 (results not shown), 5 (*b*, *e*) or 25 (*c*, *f*) mg/kg] by gavage, and killed after 4 days. Antisera to *P*450IVA (*a–c*) and ACO (*d–f*) were used to immunostain liver sections, as described in the Materials and methods section. Results are representative of two rats per treatment group.

quirement for insertion of a prosthetic group into the newly synthesized proteins.

Whereas the ACO mRNA has sequence similarity to only one gene [28] and is therefore detected with high specificity by Northern hybridization analysis, the *P*450IVA family is highly complex [11], and several members of the family are expressed in liver [29,30]. Northern hybridization analysis with a cDNA is clearly inadequate to detect a particular member of the *P*450IVA1 subfamily, as there are regions of high sequence similarity between subfamily members. However, the recently published sequences of *P*450IVA2 and *P*450IVA3 [29,30] show low sequence similarity with bases 1–39 of *P*450IVA1, thereby explaining the high induction of *P*450IVA1 noted with the oligonucleotide probe to *P*450IVA1 (Fig. 1), compared with estimates using a cDNA probe [11].

RNAase protection analysis has been shown to detect > 50% of single base pair mismatches [31]; using a 515 bp (3′ non-coding) probe, there is therefore a 97% chance of discriminating between *P*450IVA1 and a member of the subfamily which is 1% divergent. It is therefore likely that the assay reflects an absolute specificity for *P*450IVA1 mRNA. In agreement with this contention, the induction of *P*450IVA1 measured by RNAase protection (the present study) is 2–3-fold higher than the 5–7-fold induction measured using Northern blot analysis with a cDNA probe ([11]; D. R. Bell, unpublished work).

This study of the kinetics of induction of the two mRNAs by peroxisome proliferators reveals that *P*450IVA1 mRNA is induced more rapidly than ACO mRNA, thereby demonstrating a biphasic induction response. This suggests that the induction process for the two mRNAs is either mediated by different mechanisms or modulated differentially by unknown factors.

There are clear discrepancies between the kinetics of mRNA accumulation reported herein and elsewhere [11,14–16]. Pharmacokinetic considerations, such as the route of administration, site of intraperitoneal injection and the vehicle, may alter the time course of induction. These concerns are best addressed in an *in vitro* hepatocyte system, where pharmacokinetic considerations are obviated. Another confounding factor is the peroxisome proliferator itself. Clofibrate and diethylhexylphthalate have been used [11,14], as well as ciprofibrate [14] and nafenopin [16]. In view of the structural diversity and range of potencies of peroxisome proliferators, study of the kinetics of mRNA accumulation after administration of structurally diverse proliferators seems warranted.

The differential time course of induction of *P*450IVA1 and ACO mRNAs suggested the possibility that different mechanisms might mediate the induction of the peroxisomal and microsomal enzymes. We therefore decided to test whether these proteins were induced in the same regions of the hepatic lobular architecture. Recent work has demonstrated that hepatocytes *in vitro*

show differential sensitivity to *P*-450 inducers [17], and that *P*-450 IIBs are induced in a sub-population of hepatocytes by the *P*-450 inducer phenobarbital or the peroxisome proliferator clofibric acid [17]. We therefore treated animals with maximal (25 mg/kg) and sub-maximal (2 mg/kg) inducing doses of methylclofenapate, and assayed the lobular distribution of induction of ACO and cytochrome *P*450IVA. Our current experiments (Fig. 5) clearly demonstrate a dose-dependent centrilobular induction of both ACO and cytochrome *P*450IVA. Further, immunostaining of serial sections of treated rat livers revealed that both proteins were at elevated levels in an identical region of the liver lobule, demonstrating that *P*450IVA and ACO are induced in the same, spatially distinct, hepatocytes. The demonstration of a discrete hepatocyte population with enhanced sensitivity to the induction of *P*450IVA and ACO suggests that the factors which mediate the mechanism of induction are localized to specific regions of the liver lobule. Further, other phenomena associated with peroxisome proliferation may likewise show lobular localization of response. In this regard, Styles *et al.* [3] have shown that the hyperplastic response to methylclofenapate in rat liver is restricted to binucleated hepatocytes. It is currently unclear whether the hepatocyte population which undergoes S-phase following methylclofenapate treatment is coincidental with that which responds by induction of peroxisomal and microsomal enzymes.

In conclusion, we have shown that LAH and ACO activities are unchanged during the first 8 h after treatment with methylclofenapate, and that the amount of *P*450IVA1 (but not of ACO) mRNA had increased as early as 6 h after treatment, thereby demonstrating differing kinetics of induction of the microsomal and peroxisomal mRNAs. However, immunocytochemical localization of *P*450IVA and ACO within the liver lobule of methylclofenapate-treated rats revealed that these proteins were induced in a dose-dependent manner in the same spatially distinct sub-population of hepatocytes.

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