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Induction of acyl-CoA oxidase and cytochrome P450IVA1 RNA in rat primary hepatocyte culture by peroxisome proliferators

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We have characterized the induction of acyl-CoA oxidase and cytochrome P450IVA1 RNAs in a primary hepatocyte culture system *in vitro*, using a sensitive and specific RNAase protection assay. Hepatocytes were cultured with a maximal inducing dose of the peroxisome proliferator clofibric acid (1 mM), or vehicle control, for 4 days, and the level of RNAs compared with the level in rats which had been treated with corn oil or clofibric acid (300 mg/kg) for 4 days. The level of acyl-CoA oxidase and P450IVA1 RNAs in 4-day-old control hepatocytes was less than 2% of that in control liver. However, the level of these RNAs in RNA from treated hepatocytes was 61% of that in liver RNA from treated rats. Hepatocytes were treated with the potent peroxisome proliferator methylclofenapate (100 μM), and the induction of RNAs determined at various times after exposure. P450IVA1 RNA was significantly induced 1 h after dosing, rising to 34-fold above control after 8 h, whereas acyl-CoA oxidase RNA was not significantly induced until 4 h, increasing to 5.2-fold above control after 8 h. A similar time course of induction was seen after treatment of hepatocytes with 100 μM-nafenopin, 100 μM-methylclofenapate, 1 mM-clofibric acid or 1 mM-mono(ethylhexyl) phthalate, suggesting that the differential time course of induction of P450IVA1 and acyl-CoA oxidase RNAs is not related to the esterification, structure or potency of the peroxisome proliferator, but is intrinsic to the process of peroxisome proliferation. Hepatocytes were treated with methylclofenapate in the presence and absence of cycloheximide. P450IVA1 RNA was significantly induced by methylclofenapate in the presence of cycloheximide, rising to 17-fold above control after 8 h. However, no induction of acyl-CoA oxidase RNA was detected in the presence of cycloheximide. Therefore we characterize the induction of acyl-CoA oxidase and P450IVA1 RNAs in primary hepatocyte culture *in vitro* as a faithful model of the induction response in rat liver, and suggest that induction of P450IVA1 RNA is a primary event in the process of peroxisome proliferation.

INTRODUCTION

The peroxisome proliferators constitute a class of chemicals which cause induction of cytochrome *P*-450 [1], proliferation of peroxisomes [2], induction of S-phase [3] and carcinogenesis in the rodent liver [2]. The biochemical mechanisms whereby these changes occur are currently poorly understood [4], and so attention has focused on the delineation of early events in the induction process mediated by peroxisome proliferators. Clearly the definition of an early event whose induction occurs in the absence of protein synthesis will allow molecular analysis of the system mediating induction by peroxisome proliferators.

Cytochrome P450IVA1 is a fatty acid hydroxylase induced after treatment with peroxisome proliferators [5]. The rat cDNA has been cloned [6], and induction of cross-hybridizing mRNA shown to occur as early as 3 h after treatment with the peroxisome proliferator clofibrate (CFA) [6]. Enzymes of peroxisomal β-oxidation, such as acyl-CoA oxidase (ACO) and the bifunctional enzyme enoyl-CoA hydratase (3-hydroxyacyl-CoA dehydrogenase) (BFE), are also induced during peroxisome proliferation, and the cDNAs for these enzymes have been cloned [7,8]. Further work has shown that the induction of these RNAs is mediated by a transcriptional process *in vivo* [9]. The time course of induction of peroxisomal enzyme RNAs is currently confused [9,11], although this may be a consequence of the administration of different peroxisome proliferators by different routes.

Our previous studies used RNAase protection assay to compare the time course of induction of P450IVA1 and ACO RNAs in groups of rats treated with the potent peroxisome proliferator methylclofenapate [11]. These experiments demonstrated that, *in vivo*, P450IVA1 RNA was induced before elevation in ACO RNA, and moreover, that no induction of ACO RNA was apparent up to 8 h after treatment of rats [11].

The study of primary events in peroxisome proliferation may be facilitated by the use of a system where pharmacokinetic effects are minimized, such as primary hepatocyte culture *in vitro* [12]. Rat primary hepatocyte cultures have previously been shown to support induction of peroxisomal enzymes [12] and their respective RNAs [13,14]. However, the induction of P450IVA1 RNA and the magnitude of the induction compared with the '*in vivo*' situation in this system are uncharacterized. The distinct kinetics of induction of ACO and P450IVA1 RNAs *in vivo* [11], as well as the notoriously poor induction of cytochrome *P*-450 RNAs (e.g. P450IIB [15]) in cultured hepatocytes, would suggest that analysis of both RNAs would be a prerequisite for establishing primary hepatocyte culture as a faithful model of induction in the intact liver.

We have therefore used RNAase protection assay to compare the absolute levels of induced ACO and P450IVA1 RNAs in the liver and in rat primary hepatocyte cell culture and to delineate the induction kinetics of both RNAs. Further, we have analysed the effect of structurally dissimilar peroxisome proliferators with differing potencies on the induction of ACO and P450IVA1 RNAs and have determined the inducibility of these RNAs in the presence of the protein synthesis inhibitor cycloheximide.

Abbreviations used: P450IVA1, cytochrome *P*-450 species P450IVA1; ACO, acyl-CoA oxidase; BFE, enoyl-CoA hydratase; poly(A)$^+$, polyadenylate(d); CFA, clofibric acid; MEHP, mono(ethylhexyl) phthalate.

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MATERIALS AND METHODS

Materials

Methylclofenapate was synthesized by Lancaster Synthesis, Morecambe, Lancs., U.K., CFA was from Sigma, Nafenopin was kindly given by Dr. Bentley of Ciba–Geigy, and all other chemicals were of the highest grade available.

Dosage with proliferators

Male Alderley Park (Alpk: AP$_f$SD) rats (180–220 g) were dosed by gavage with 300 mg of CFA/kg body weight in a volume of 5 ml of corn oil/kg, phenobarbital in saline (80 mg/kg), or corn-oil vehicle control, for 4 consecutive days. Food and water was provided *ad libitum*. Rats were killed by exsanguination after halothane anaesthesia, and the livers were removed and frozen in liquid N_2. Naïve rats were killed with diethyl ether and the livers perfused, hepatocytes isolated, seeded and replenished after 4 h with CL15 medium exactly as described in [12]. Treatment of hepatocytes was by two protocols.

Protocol A. Duplicate groups of four plates of cells were dosed with 1 mM-CFA, 0.25% dimethylformamide in CL15 or vehicle control at 16 h after seeding, and the medium (containing CFA or vehicle) was replaced each day for 4 days.

Protocol B. Cells were fed with CL15 medium at 16 h after seeding, and were dosed 24 h later with 100 μM-methylclofenapate, 1 mM-CFA, 100 μM-nafenopin, 1 mM-mono (ethylhexyl) phthalate (MEHP) or vehicle control with or without cycloheximide (30 μg/ml), in 0.25% dimethylformamide in CL15 medium. Cells were harvested at the indicated time. The experiments described in Table 1 and Fig. 3 (below) were performed with triplicate groups of four plates of cells per time point, whereas the results in Fig. 2 (below) were obtained with one set of pooled plates per point.

Isolation and analysis of RNA

RNA was isolated from liver by guanidine thiocyanate/CsCl methodology, exactly as described in [11]. Hepatocytes were washed with ice-cold saline (0.9% NaCl), and then lysed in chilled guanidine thiocyanate solution, followed by centrifugation over CsCl, exactly as described in [11]. RNAs were electrophoresed through an agarose gel and stained to demonstrate integrity of 28 S and 18 S ribosomal RNA bands, and were then normalized by hybridization in triplicate to ^{32}P-labelled oligo(dT)$_{18}$ and liquid-scintillation counting [17]. Plasmids pIV2 (P450IVA1) and pACO.227 (ACO) were described previously [11] and were linearized with restriction endonucleases *Alu*I and *Bam*HI respectively, for transcription of antisense RNA probes, RNAase protection and densitometry as described in [11]. The lengths of protected probe fragments were 515 bp and 227 bp for P450IVA1 and ACO RNA respectively. RNAase protection analysis was performed twice on each RNA sample.

Statistics

Analysis of statistical significance was performed using Students t test relative to concurrent controls, and $P < 0.05$ was deemed to be significant.

RESULTS

Absolute levels of ACO and P450IVA1 RNAs *in vivo* and *in vitro*

RNA samples were isolated from the liver of rats treated with 300 mg/kg of CFA [18], phenobarbital or corn oil vehicle for 4 days, and from hepatocytes treated with 1 mM-CFA or vehicle control for 4 days, and were normalized for polyadenylate [poly(A)$^+$] content [17]. RNAase protection on these samples was assayed using both the ACO and P450IVA1 probes in the same hybridization, and a typical autoradiograph is shown in Fig. 1. After compensation for probe length and specific activity, ACO RNA is present at a higher level than P450IVA1 in control rat liver RNA and also in liver RNA of rats treated with CFA. ACO RNA is also at a higher level than P450IVA1 RNA in RNA from control or CFA treated hepatocytes.

Comparison of the relative amount of ACO or P450IVA1 RNAs in RNA from control liver and from 4-day-old hepatocyte cultures revealed that the constitutive steady-state levels of P450IVA1 or ACO RNA *in vitro* are approx. 2% of the level in liver. However, comparison of the amounts of ACO or P450IVA1 RNA between RNA from the liver of CFA treated rats or from hepatocyte culture showed that these RNAs were present *in vitro* at 61% of the concentration *in vivo*. Thus, although the induction of ACO and P450IVA1 RNAs *in vivo* is in the range of 11–18-fold ([11]; Fig. 1), the induction of these RNAs *in vitro* is 500-fold (Fig. 1), primarily because of the decrease in constitutive levels of these RNAs.

Induction kinetics of ACO and P450IVA1 RNAs *in vitro*

Hepatocytes were treated with 100 μM-methylclofenapate at 44 h after seeding, and triplicate sets of four pooled plates of hepatocytes were lysed at the indicated time and analysed in duplicate by RNAase protection. Treatment of hepatocytes with methylclofenapate at 16 h after seeding gave similar results (results not shown). Simultaneous hybridization to the ACO and P450IVA1 RNA probes was assayed to obviate the effect of

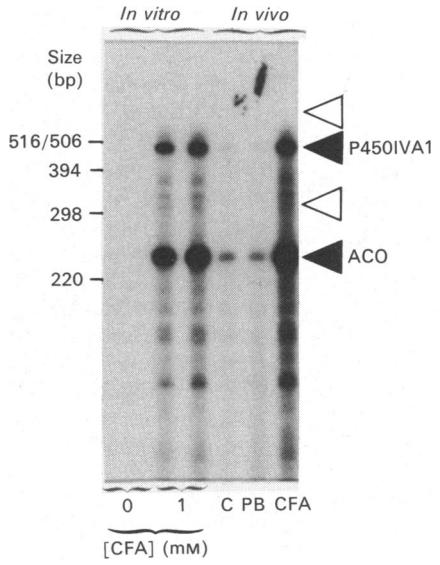

Fig. 1. Constitutive and inducible expression of ACO and P450IVA1 RNAs *in vivo* and *in vitro*

In vivo: rats were treated with corn oil(C), phenobarbital(PB) or clofibric acid (CFA) for 4 days as described in the Materials and methods section, and livers were removed for RNA analysis. *In vitro*: hepatocytes were isolated from naïve rats, and duplicate groups of four plates were treated with CFA (1 mM-CFA) or vehicle (0 mM-CFA) for 4 days under protocol A as described in the Materials and methods section. RNA was isolated, normalized by hybridization to oligo(dT)$_{18}$, and 30 μg of each RNA sample was hybridized to the ACO and P450IVA1 antisense probes for simultaneous RNAase protection assay, as described in the Materials and methods section. The open triangles indicate the position of unprotected probe, and the closed triangles represent the position of the protected probe fragments (515 bp for P450IVA1 and 227 bp for acyl-CoA oxidase).

Table 1. Induction of P450IVA1 and ACO RNA in primary hepatocyte culture

Hepatocytes were treated under protocol B with methylclofenapate (M, 100 μM) or vehicle (C, 0.25% dimethylformamide), and were removed at the indicated time after dosing. RNA was isolated from triplicate groups of four pooled plates of hepatocytes, and RNA assayed in duplicate by simultaneous RNAase protection assay for ACO and P450IVA1, as described in the legend to Fig. 1. Results are presented relative to control values for ACO or P450IVA1 RNA at zero time, which are given an arbitrary value of 1, and results which are significantly different at $P < 0.05$ from time-matched controls are in *italics*. Results are means ± S.D. for three samples.

		ACO RNA			P450IVA1 RNA		
Treatment	Time (h)	Mean	S.D.	Induction (fold)	Mean	S.D.	Induction (fold)
C	0	1	0.481	–	1	0.754	–
C	0.5	1.314	0.222	–	1.030	0.085	–
M	0.5	1.289	0.071	0.981	1.321	0.500	1.28
C	1	1.464	0.085	–	1.307	0.385	–
M	1	1.624	0.253	1.109	*1.965*	*0.126*	*1.50*
C	2	1.216	0.493	–	1.452	0.401	–
M	2	2.036	0.255	1.674	*5.495*	*1.593*	*3.78*
C	4	1.448	0.190	–	1.586	0.107	–
M	4	*3.149*	*0.078*	*2.18*	*19.0*	*0.645*	*12.0*
C	8	1.510	0.092	–	1.853	0.296	–
M	8	*7.780*	*0.129*	*5.152*	*63.9*	*16.0*	*34.5*

sample-handling errors during analysis of comparative induction kinetics. Table 1 shows the time course of P450IVA1 RNA induction; although there is no significant change at 0.5 h, the RNA level increased significantly to 1.5-fold at 1 h after treatment with methylclofenapate, rising to 34-fold above control 8 h after dosing. Table 1 shows the time course of induction of ACO RNA. By contrast with the early induction of P450IVA1 RNA, ACO RNA was not significantly induced until 4 h after treatment (2.2-fold), and rose to 5.2-fold above control after 8 h.

RNA induction kinetics with different peroxisome proliferators

Hepatocytes were treated with either 1 mM-CFA, 1 mM-MEHP, 100 μM-nafenopin, 100 μm-methylclofenapate or vehicle control, and groups of four plates were harvested for RNA isolation and analysis at the indicated time after dosing (Figs. 2*a* and 2*b*). P450IVA1 RNA was increased 1.5–3-fold above control in the samples at 2 h after dosing, rising to 31–77.8-fold above control at 12 h after dosing (Fig. 2*a*). By contrast, the level of ACO RNA was unperturbed at 2 h after dosing (0.76–1.17), but increased to 1.4–2.6-fold above control at 4 h after dosing (Fig. 2*b*). The induction at 12 h varied from 14–24-fold above control. Thus, for structurally dissimilar peroxisome proliferators of differing potencies, induction of P450IVA1 precedes the induction of ACO RNA, even though the fold induction of RNAs at 12 h was less for the less potent proliferator, MEHP.

Induction of P450IVA1 RNA in the presence of cycloheximide

Hepatocytes were treated with 100 μM-methylclofenapate or vehicle control in the presence or absence of cycloheximide (30 μg/ml). Triplicate groups of four pooled plates of hepatocytes were taken for RNA analysis at the indicated time after induction (Fig. 3). At 2 h after dosing, P450IVA1 RNA was significantly increased 2.3 (methylclofenapate)- and 3.1 (methylclofenapate and cycloheximide)-fold above control, rising to 91 (methylclofenapate)- and 17 (methylclofenapate and cycloheximide)-fold above control at 8 h (Fig. 3*a*). However, in cells treated with methylclofenapate and cycloheximide, the absolute level of P450IVA1 did not increase between 4 and 8 h after dosing; this may reflect the loss of a short-lived *trans*-acting factor necessary for transcription as a consequence of cycloheximide treatment. There was no detectable deterioration of morphology in hepatocytes exposed for 8 or 12 h to cycloheximide (results not shown).

ACO RNA increased significantly to 1.9-fold above control at 4 h hours, and rose to 8.6-fold above control at 8 h, after treatment of cells with methylclofenapate (Fig. 3*b*). ACO RNA levels in cells treated with cycloheximide or methylclofenapate and cycloheximide were indistinguishable from control values. Since the bulk of induction of ACO RNA does not occur until after 4 h (Table 1; Fig. 2*b* and 3*b*), ablation of induction of this RNA by cycloheximide may reflect the same toxicity seen with P450IVA1 RNA after 4 h treatment with cycloheximide. Consequently, we conclude that induction of P450IVA1 RNA by methylclofenapate occurs in the presence of protein-synthesis inhibitors, but, in contrast, we found no evidence for induction of ACO RNA in the presence of cycloheximide.

DISCUSSION

We have examined the induction of ACO and P450IVA1 RNAs both *in vitro* in primary cultures of rat hepatocytes and *in vivo* in the rat liver. CFA was used as inducing agent in a comparison of induction *in vivo* and *in vitro*, as the maximal inducing dose of this compound has been previously characterized ([18]; R. G. Bars, D. R. Bell & C. R. Elcombe, unpublished work). Steady-state levels of ACO and P450IVA1 RNAs in 4-day-old control cultured hepatocytes are much lower (~2%) than the level of these RNAs in RNA from control rat liver (Fig. 1). The decline in the constitutive levels of these RNAs may be due to loss of *trans*-acting factors, owing to dedifferentiation or to a lack of some endogenous inducer, but it is also possible that previously characterized mechanisms, such as growth-hormone regulation of P450IIB species [20,21], may control the levels of these RNAs *in vitro*. However, when we examined the amount of both ACO and P450IVA1 RNAs in RNA from CFA-treated hepatocyte or liver RNA, the level of these RNAs *in vitro* was 61% of the level *in vivo* (Fig. 1). It is therefore apparent that the inducibility of these RNAs does not require maintenance of the 'constitutive' levels of RNA, and that the maximally induced levels of these RNAs *in vitro* approach the magnitude seen *in vivo*.

The structurally related and more potent CFA analogue

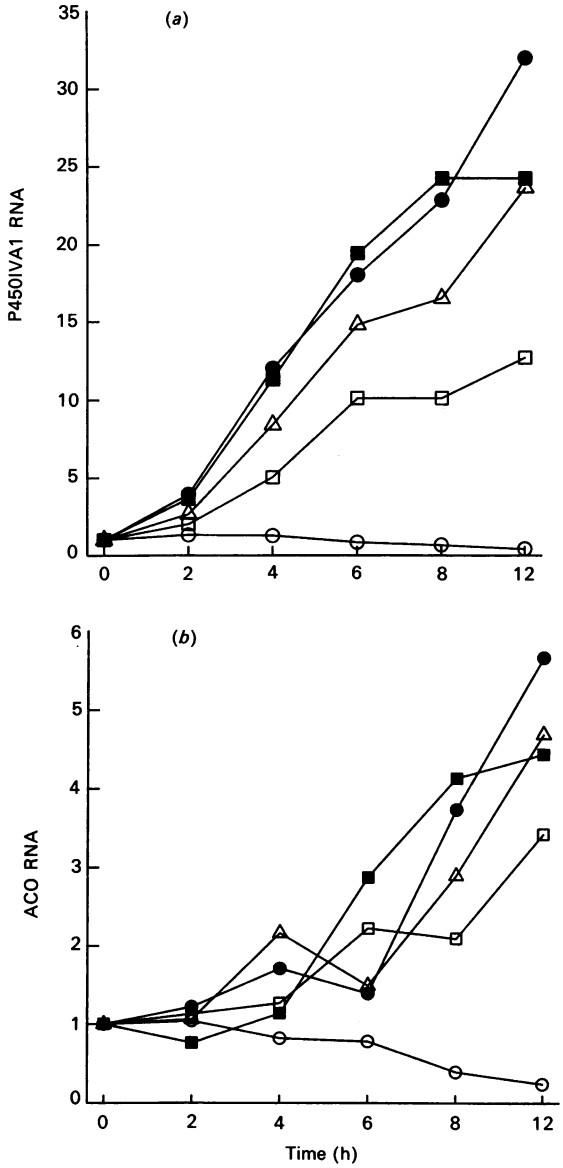

Fig. 2. Induction of P450IVA1 (*a*) and ACO (*b*) RNA by peroxisome proliferators

Hepatocytes were treated under protocol B with 100 μM-methylclofenapate (●), 100 μM-nafenopin (■), 1 mM-CFA (△), 1 mM-MEHP (□) or vehicle control (○), and groups of four plates of hepatocytes were pooled for RNA analysis at the indicated time after dosing. RNAase protection was performed as described in the legend to Table 1. RNA is expressed in arbitrary units (see Table 1).

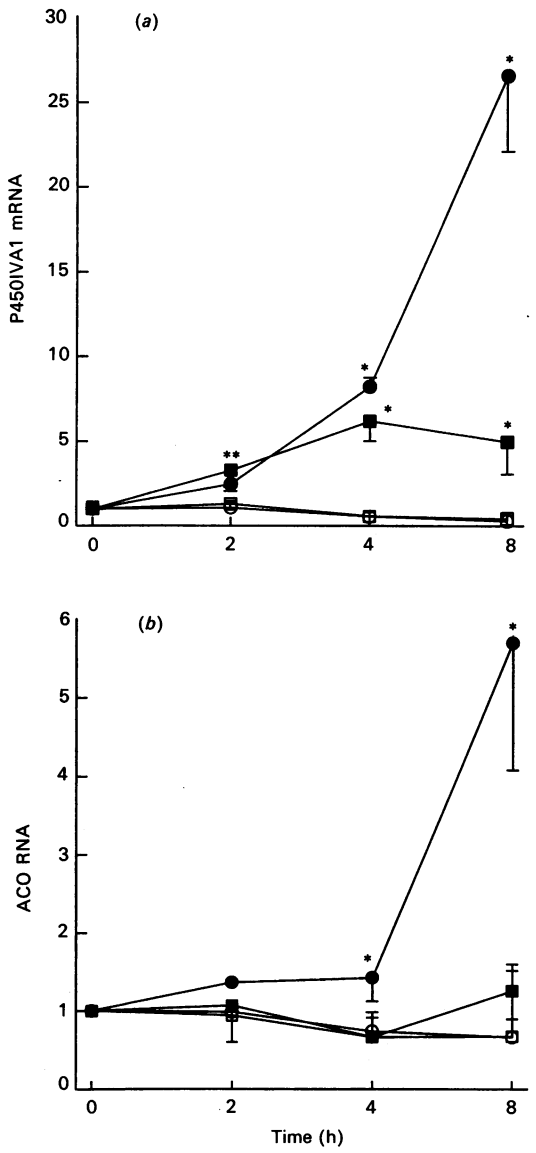

Fig. 3. Induction of P450IVA1 (*a*) and ACO (*b*) RNA in the presence of cycloheximide

Triplicate groups of four plates of hepatocytes were treated with 100 μM-methylclofenapate (●), 100 μM-methylclofenapate and cycloheximide (30 μg/ml) (■), vehicle (○) or cycloheximide (30 μg/ml) and vehicle (□), and RNA isolated and analysed by RNAase protection assay at the indicated time after treatment, as described in the legend to Table 1. Results which are significantly different at $P < 0.05$ from concurrent controls are indicated by an asterisk. Results are means ± S.D. ($n = 3$). RNA is expressed in arbitrary units (see Table 1).

methylclofenapate was used for analysis of induction kinetics in order to determine comparability with previous studies *in vivo* [11]. Induction of P450IVA1 RNA consistently preceded the induction of ACO RNA in primary hepatocytes treated with methylclofenapate (Table 1; Figs 2 and 3) by 2–3 h. The simultaneous use of both probes in an RNAase protection assay obviates the possibility of artefacts due to sample handling, and statistical analysis of normalized protection data was undertaken to indicate significance. Induction of P450IVA1 and ACO RNAs in hepatocytes (the present study) occurs 4–5 h earlier than the corresponding increase *in vivo* [11]. The earlier induction response *in vitro* may relate to the immediate bioavailability of methylclofenapate in culture, as opposed to the pharmacokinetics of delivery of intraperitoneally dosed methylclofenapate to the liver [11], and suggests that induction kinetics of RNA after exposure to methylclofenapate may be similar *in vitro* and *in vivo*.

Thus, the data obtained *in vitro* described herein are consistent with the differential kinetics of RNA induction of P450IVA1 and ACO RNA described after administration of methylclofenapate *in vivo* [11], but fail to explain the early induction (at 1 h) of ACO RNA after treatment of Fisher 344 rats with various proliferators [9]. Possible explanations for the difference in RNA induction kinetics in [9] and [11] include pharmacokinetic considerations, such as route of administration of compound, or the use of different peroxisome proliferators. In particular, methylclofenapate is the methyl ester of clofenapic acid, and may

require metabolism (hydrolysis) to an active form. Therefore we treated hepatocytes with each of four peroxisome proliferators in order to compare the resulting induction kinetics of ACO and P450IVA1 RNA. As shown in Fig. 2, we were unable to differentiate the induction kinetics of ACO and P450IVA1 RNAs on the basis of proliferator potency (nafenopin and MEHP), esterification status (nafenopin versus methylclofenapate) or structure (clofibric acid and MEHP). All compounds tested caused a more rapid induction of P450IVA1 RNA compared with ACO RNA, at the same time after treatment. It is therefore difficult to reconcile the reported differences in induction kinetics *in vivo* with the choice of peroxisome proliferator; however, different rat strains were employed in these analyses [9,11].

Moreover, we have consistently observed that induction of P450IVA1 RNA precedes induction of ACO RNA after methylclofenapate treatment, either *in vivo* or *in vitro*. Further, treatment of hepatocytes with each of four different peroxisome proliferators resulted in P450IVA1 RNA induction preceding that of ACO RNA (Fig. 2), suggesting that differential induction of P450IVA1 and ACO RNAs may be an inevitable consequence of treatment with peroxisome proliferators. The differential induction kinetics of these RNAs may reflect different mechanisms controlling the induction process for microsomal or peroxisomal enzyme RNAs or, alternatively, the absolute levels of P450IVA1 and ACO RNAs may determine the time at which induction is detectable.

Therefore induction of P450IVA1 and acyl-CoA oxidase RNAs was examined in the presence or absence of the protein-synthesis inhibitor cycloheximide (Fig. 3). Cycloheximide is known to rapidly inhibit protein synthesis in hepatocytes (see, e.g., [22]). Although P450IVA1 was significantly inducible in the presence of cycloheximide and methylclofenapate, rising to 17-fold above control at 8 h, we found no evidence for induction of ACO RNA by methylclofenapate in the presence of cycloheximide. These findings are consistent with the model previously proposed by Elcombe & co-workers [4], which postulates that cytochrome P450IVA1 triggers the induction of peroxisomal enzymes, but are also compatible with other mechanisms, such as induction of a *trans*-acting factor which is necessary for induction of acyl-CoA oxidase RNA. Thus we define induction of P450IVA1 RNA as a primary event in the peroxisome-proliferation response. Since induction of P450IVA1 RNAs in rat liver is known to occur by a transcriptional activation [6], we postulate that activation of the P450IVA1 gene is mediated by a *trans*-acting factor which functions as a pleiotropic effector in the peroxisome-proliferator response.

We have demonstrated that primary hepatocyte culture *in vitro* is a faithful model of the peroxisome proliferation RNA induction response seen *in vivo*, even though the levels of ACO and P450IVA1 RNAs in control hepatocyte cultures is dramatically reduced compared with that seen in RNA from control rat liver. We have demonstrated differential kinetics of induction of the P450IVA1 and ACO RNAs after treatment of hepatocytes with structurally dissimilar peroxisome proliferators of differing potency, and we have shown that induction of the P450IVA1 RNA is a primary event in the peroxisome-proliferation response. This model should facilitate the analysis of functional elements in the P450IVA1 gene, and expedite understanding of the mechanism of action of peroxisome proliferators.

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