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INDUCTION OF CYTOCHROME P450 AND PEROXISOMAL ENZYMES BY CLOFIBRIC ACID *IN VIVO* AND *IN VITRO*

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Abstract—We have analysed the induction of microsomal and peroxisomal proteins and their RNAs after treatment of hepatocytes with the peroxisome proliferator, clofibric acid, *in vitro* and *in vivo*. After treatment of hepatocytes with 1 mM clofibric acid for 4 days, P450 4A1 RNA is induced 500-fold, and acyl-CoA oxidase and P450 2B1 280-fold, relative to control cultures. These RNAs are detectably induced after administration of 25 μM clofibric acid, and show a similar induction response with increasing doses of clofibric acid. Western blot analysis of the P450 4A and bifunctional enzyme (BFE) proteins showed that both were induced in parallel with increasing doses of clofibric acid, over a range of 25 μM–1 mM. The distribution of the induced proteins was examined by immunocytochemistry. Increasing doses of clofibric acid led to an increase in the average intensity of staining for both proteins throughout the hepatocyte population. There was, however, a graded variation between hepatocytes in the intensity of staining, both for P450 4A and BFE proteins. The heterogeneity in response of the hepatocyte population *in vitro* may be related to differential sensitivity of hepatocytes to induction *in vivo*. Therefore, rats were dosed with 0, 50 or 300 mg/kg of clofibric acid for 4 days by gavage, and the livers were examined by immunocytochemistry. After 50 mg/kg of clofibric acid, both P450 4A and BFE were induced mainly in zones 3 and 2 of the liver acinus. However, after 300 mg/kg of clofibric acid, staining for both proteins was strong and homogenous throughout the liver acinus. Thus, hepatocytes from zones 3 and 2 of the acinus are differentially responsive to induction by clofibric acid.

The peroxisome proliferators are a structurally diverse group of chemicals, which were originally characterized on the basis of the peroxisome proliferation and associated tumourigenicity of these chemicals in rodent liver [1, 2]. Subsequent work has shown that peroxisome proliferators activate a pleiotropic response in the rodent liver, including induction of peroxisomal proteins and their RNAs [3, 4], proliferation of the smooth endoplasmic reticulum, induction of cytochromes P450 4A [5, 6] and corresponding RNAs [7, 8], and an acute hyperplastic response [9]. A novel member of the steroid hormone receptor superfamily has recently been shown to be activated by peroxisome proliferators [10]. Although the function of this protein is not known, it is possible that it may mediate part, or all, of the peroxisome proliferation response.

Induction of cytochromes P450 4A and peroxisomal β-oxidation enzymes [e.g. acyl-CoA oxidase, enoyl-CoA hydratase: 3-hydroxyacyl-CoA dehydrogenase or bifunctional enzyme (BFE§)] is an early consequence of treatment with peroxisome proliferators [3, 4, 7, 8], and these enzymes are markers of the peroxisome proliferation response. Enzymes of β-oxidation and their RNAs are known to be induced in parallel by several peroxisome proliferators [11]. Comparison of the induction of the microsomal laurate hydroxylase (cytochrome P450 4A) and peroxisomal enzymes showed a parallel dose–response [12, 13]. However, recent studies [14] have shown that P450 4A1 RNA is induced before acyl-CoA oxidase RNA; this suggests that the induction of RNAs encoding microsomal and peroxisomal enzymes may not be controlled by the same mechanism. More detailed studies to delineate the dose–response for induction of microsomal and peroxisomal proteins and their corresponding RNAs may help to address the coordinate regulation of these two systems.

The study of enzymes and RNA in organ homogenates has provided insight into the mechanisms of induction, but not an analysis of the cellular targets which respond to peroxisome proliferators. Bars *et al.* [15] used *in vitro* techniques to show that hepatocytes in culture respond heterogenously to phenobarbital treatment, as measured immunocytochemically by induction of P450 2B. The heterogenous response of hepatocytes to phenobarbitone treatment is mirrored *in vivo*, where P450 2B is preferentially induced in zones 2 and 3 of the liver acinus [16]. In this light, the heterogenous induction of P450 2B immunoreactive proteins by the peroxisome proliferator, clofibric acid [15], and the description of cell populations which are differentially sensitive to the induction of S-phase by the peroxisome proliferator, methylclofenapate [9], suggested that the induction of cytochromes P450 4A and peroxisomal enzymes warranted further investigation. Moreover, the demonstration of induction of P450 2B family proteins by the prototype

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§ Abbreviations: BFE, bifunctional enzyme; pcr, polymerase chain reaction.

P450 4A inducer, clofibric acid [15], clearly merits further investigation. We have therefore examined the *in vitro* induction response of microsomal and peroxisomal proteins and RNAs with increasing doses of clofibric acid. Moreover, we have used immunocytochemical techniques to investigate the heterogeneity of this response, both *in vivo* and *in vitro*, to different doses of clofibric acid.

MATERIALS AND METHODS

Animals. Male Alderley Park (Alpk:AP^{f}SD) rats (180–220 g) were dosed by gavage with 0, 50 or 300 mg/kg of body weight of clofibric acid (Sigma Chemical Co., Poole, U.K.), in a volume of 5 mL of corn oil, or 100 mg/kg of phenobarbitone in saline, for 4 consecutive days. Food and water were provided *ad lib.* Three rats per group were killed by exsanguination after halothane anaesthesia, the livers were fixed and immunocytochemistry was performed, as described [14].

Hepatocyte culture. Naive rats were killed with ether, and the livers were perfused for hepatocyte isolation exactly as detailed [17]. Leibowitz 15 culture medium (Gibco, Uxbridge, U.K.) was supplemented with foetal bovine serum (8.3%), tryptose phosphate broth (8.3%), penicillin G (41.3 U/mL), streptomycin (8.2 μm/mL), glutamine (0.24 mg/mL), insulin (1 μM) and hydrocortisone (10 μM). Hepatocytes were dosed with 0–1 mM clofibric acid in dimethylformamide vehicle (final 0.25% v/v), at 24 hr after seeding, and the medium was replaced every day for 4 days. Hepatocyte samples for immunocytochemistry or western blot analysis were taken as described previously [15].

RNA analysis. RNA samples were prepared from 8×10^6 hepatocytes (four pooled plates) by guanidinium thiocyanate lysis and CsCl centrifugation [14]. RNA integrity was assessed by agarose gel electrophoresis, and normalization of samples was by hybridization to oligo (dT) [18]. Plasmids pIV2 (P450 4A1) and pACO.227 (acyl-CoA oxidase) have been described [14]. The 2B1 probe was transcribed from a polymerase chain reaction (pcr) template; oligos for pcr spanned the region of 967–1038 bp of 2B1 and were 5′TAATACGACTCACTATAGATCGATCAGG TGATCGGCTCACAC3′ and 5′ATTTAGGTGAC ACTATAGAATCTTGGGAAGCAGGTACCCTC GGA3′, used with a rat 2B1 cDNA (a kind gift of Dr Anderson) and cycled 15 times at: 94°, 1 min; 60°, 2 min; 72°, 1 min. Of the pcr product 10% was treated with proteinase K, extracted with phenol/$CHCl_3$ and used for transcription with SP6 polymerase as described [14]. RNAse protection assay was exactly as in Ref. 14, with the exception that hybridization was at 45° and RNAse T_1 was omitted from the RNAse digestion. Autoradiographs were analysed with a laser densitometer, in the linear range of film response.

RESULTS

Coinduction of acyl CoA oxidase P450 4A1 and P450 2B1 RNAs in vitro

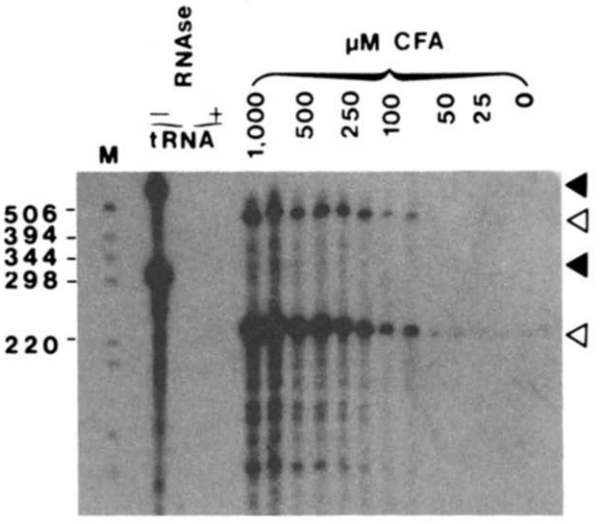
Primary cultures of rat hepatocytes were treated for 4 days with 0–1 mM clofibric acid (higher doses of clofibric acid are cytotoxic, R.G.B., unpublished), and samples collected for RNA and protein analysis. Two RNA samples (each from four pooled plates) were analysed by simultaneous RNAse protection assay for acyl-CoA oxidase and P450 4A1 RNAs, Fig. 1a. Both the acyl-CoA oxidase and P450 4A1 RNA probes were incubated with yeast tRNA throughout the reaction procedure in the absence of RNAse; these probes were full-length, indicating that the synthesis reaction gave a high proportion of full-length probe and that the reagents were free of adventitious nuclease contamination. However, when these probes were hybridized to yeast tRNA and incubated with RNAse A, no signal was visible, even after prolonged exposure (data not shown).

Very low levels of acyl-CoA oxidase and P450 4A1 RNAs are present in 4-day-old control hepatocytes, although acyl-CoA oxidase RNA is present in molar excess over P450 4A1 RNA. There is a small-fold induction of both RNAs after the lowest dose, 25 μM clofibric acid, increasing to a maximal induction of 270-fold for acyl-CoA oxidase RNA and ~500-fold for P450 4A1 RNA at 1 mM clofibric acid (Fig. 1a). In agreement with previous *in vivo* observations, acyl-CoA oxidase RNA was present at higher levels than P450 4A1 RNA.

Our previous data showed that clofibric acid treatment of primary hepatocytes induced P450 2B immunoreactive protein(s) [15]. In order to identify specific forms of P450 which are induced by clofibric acid, we examined the induction of P450 2B1 RNA with an RNAse protection assay which has been shown to differentiate between P450 2B1 and P450 2B2 RNAs (R.G.B., D.R.B., C.R.E. and Kai Lindros, manuscript in preparation). These data show that P450 2B1 is marginally induced (2-fold) by 25 μM clofibric acid, and is 280-fold above control values with 1 mM clofibric acid (Fig. 1B).

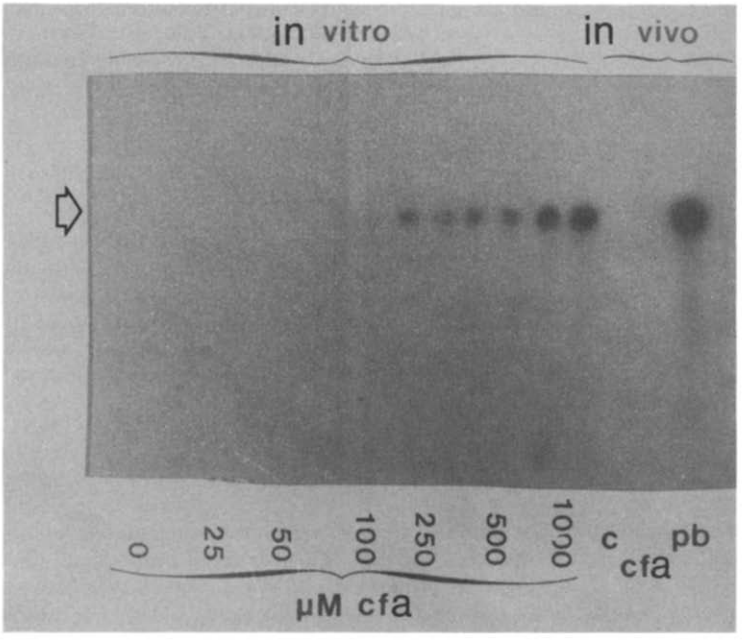
Fig. 1. Induction of RNAs by clofibric acid. (a) Induction of acyl-CoA oxidase induction (ACO) and P450 4A1 RNAs; (b) induction of P450 2B1 RNA; (c) dose–response curves for induction of ACO, P450 4A1 and P450 2B1 RNAs. Hepatocytes were treated with varying concentrations of clofibric acid (CFA) for 4 days, as described in Materials and Methods. Rats were dosed with vehicle, phenobarbital (pb) or CFA for 4 days, and the liver frozen immediately. RNA was isolated from duplicate samples of four pooled plates, normalized by hybridization to oligo (dT) and analysed by RNAse protection assay. Signals were quantified by laser densitometry in the linear range of film response. Markers (M) are end labelled 1 kb ladder (BRL). tRNA+/− represents tRNA incubated with RNA probe throughout the protection procedure in the presence (+) or absence (−) of RNAse. The closed triangles represent the position of full-length probe and the open triangles represent the position of the protected fragment (515 bp for P450 4A1, 227 bp for acyl-CoA oxidase and 172 bp for P450 2B1). (c) Acyl-CoA oxidase is represented by a square (□), P450 2B1 by a circle (○) and P450 4A1 by a triangle (△).

(a)



Dose Response to CFA: ACO and P450IVAI mRNA

(b)



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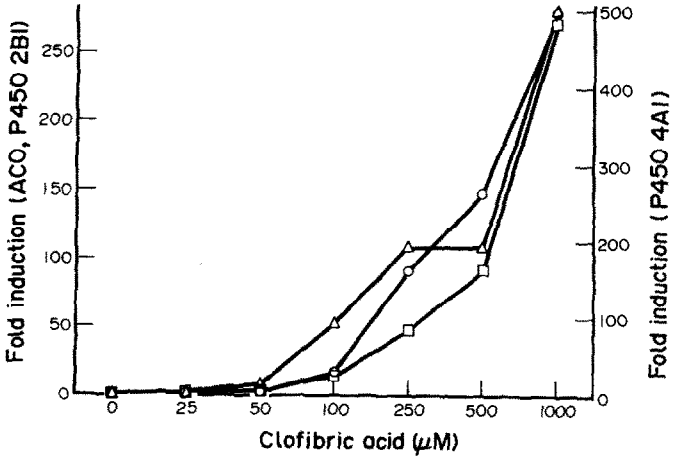

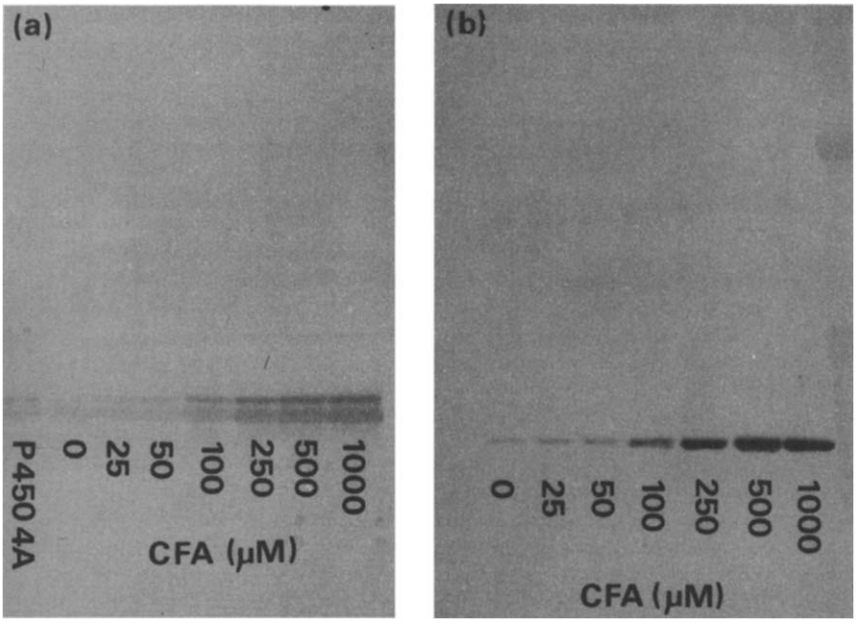

Fig. 2. Induction of peroxisomal and microsomal proteins by clofibric acid. Induction of (a) P450 4A and (b) BFE proteins. Proteins were isolated from hepatocytes treated with varying doses of clofibric acid, and western blotted with antibodies to P450 4A or BFE, as described in Materials and Methods. CFA (0–1000 μM) indicates the concentration of clofibric acid to which cells were exposed. P450 4A is a sample of purified cytochrome P450 4A, provided by Dr C. R. Wolf.

The level of P450 2B1 message in RNA from cells cultured with 1 mM clofibric acid is one third of that seen in RNA from phenobarbital-treated rat liver *in vivo* (Fig. 1B).

The data in Fig. 1a and b are shown graphically in Fig. 1c. Acyl-CoA oxidase-P450 4A1 and P450 2B1 RNAs show a similar dose–response to induction with clofibric acid.

Coinduction of P450 4A and BFE in vitro *by western blotting*

Hepatocytes from the same experiment were analysed for the presence of cytochromes P450 4A (Fig. 2a) and peroxisomal BFE (Fig. 2b) proteins by western blotting. These data show small increments in the level of both P450 4A and BFE proteins at low doses, but a more pronounced increase is between concentrations of 50 and 100 μM clofibric acid. These data therefore parallel those obtained with RNAse protection assay (Fig. 1b).

Heterogeneity of induction of P450 4A and BFE in hepatocytes in vitro

Immunocytochemical detection of P450 4A or BFE in untreated cultures of rat hepatocytes showed a slight positive staining for both enzymes in a few cells (Fig. 3a, d). When hepatocytes were exposed to clofibric acid (25 μM–1 mM) for 4 days, positive staining for both proteins was visualized in the majority of the cell population (Fig. 3b, c, e, f). The average intensity of staining in hepatocytes increased with the concentration of clofibric acid. However, there was graded variation between hepatocytes in the intensity of staining for either P450 4A or BFE throughout the range of concentrations of clofibric acid. The staining for P450 4A within single cells appeared homogeneously distributed, whereas the staining for BFE had a granular appearance, and was often distributed around the nuclei of the cells (Fig. 3e, f).

Heterogeneity of induction of P450 4A and BFE in the liver acinus

Immunohistochemical staining for P450 4A or BFE on control liver sections showed minimal positive staining all over the liver acinus, which was slightly more pronounced in acinar zone 3 (Fig. 4a, d). When animals were administered a low dose of clofibric acid (50 mg/kg), there was strong positive staining for both P450 4A and BFE in zones 2 and 3 (Fig. 4b, e). Moreover, the localization of P450 4A and BFE in consecutive serial sections revealed that the acinar distributions of the two proteins were indistinguishable. When animals were administered a high dose (300 mg/kg) of clofibric acid, strong and homogenous staining for both P450 4A and BFE was found throughout the liver acinus (Fig. 4c, f).

DISCUSSION

We have analysed the RNA induction profile of the prototype peroxisome proliferator, clofibric acid, in primary cultures of rat hepatocytes. The data in Fig. 1 reveal that P450 4A1 and acyl-CoA oxidase RNAs are highly inducible by clofibric acid *in vitro*. Moreover, this work demonstrates that the fold induction of P450 4A1 and acyl-CoA oxidase RNA in hepatocytes *in vitro* (500-fold, Fig. 1) is actually

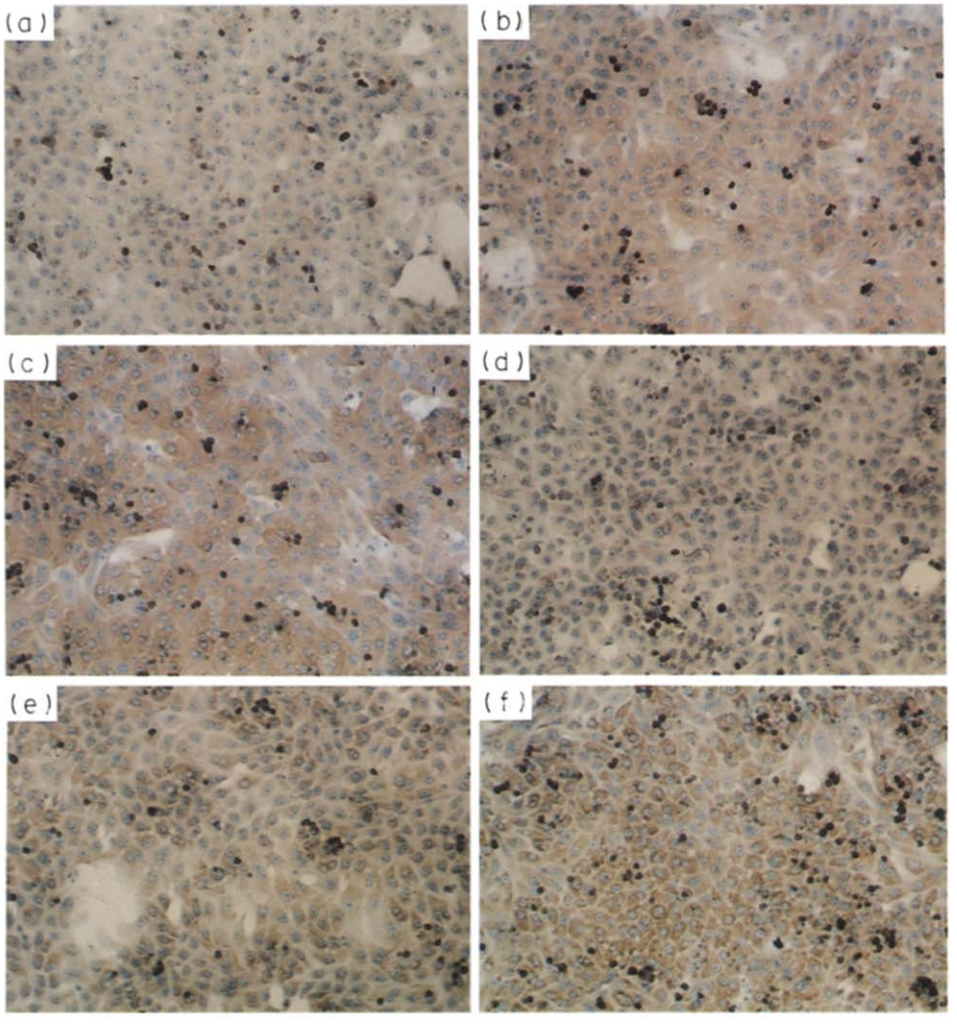

Fig. 3. Immunocytochemical localization of (a–c) P450 4A and (d–f) BFE in hepatocytes *in vitro*. Hepatocytes were cultured in the presence of 0 (a, d), 100 (b, e) or 1000 (c, f) μM clofibric acid, fixed, and immunostained for P450 4A (a, b, c) or BFE (d, e, f) as described in Materials and Methods. Similar results were seen throughout the range of doses employed, and are representative of several experiments.

greater than that achieved *in vivo* (15-fold [14]). Both RNAs are noticeably induced by 25 μM clofibric acid, demonstrating that there are significant induction effects with doses of clofibric acid 40-fold lower than the maximal inducing dose that we studied.

Bars *et al.* found that P450 2B proteins were induced to a higher level in cultured hepatocytes treated with 1 mM clofibric acid than in cells treated with phenobarbitone [15]. We used a highly specific RNAse protection assay, which discriminates between P450 2B1 and P450 2B2 RNAs (R.G.B., D.R.B., C.R.E. and K. Lindros, manuscript submitted), to demonstrate that P450 2B1 RNA is present in 4 day control cultures, and that it is induced 280-fold with 1 mM clofibric acid. By direct comparison with RNA from the liver of a phenobarbital-treated rat, this induced level *in vitro* is 32% of that seen *in vivo* (Fig. 1b). The culture conditions employed in our experiments [i.e. 17], with Falcon 3013 flasks (without substratum) and medium containing 8.3% foetal calf serum, yield greater inducibility of P450 2B1 RNA than reported previously [19]: the necessity for "Matrigel" substratum [19] or serum free conditions [20] for the maintenance of P450 2B1 inducibility must therefore be reassessed. Induction of P450 2B1 RNA in hepatocytes treated with clofibric acid *in vitro* is consistent with the recent results of Srivastava *et al.* [21], who used oligonucleotide probes to show that clofibric acid induces P450 2B1 in rat liver.

Analysis of the dose–response for induction of RNAs with clofibric acid revealed that acyl-CoA oxidase, P450 2B1 and P450 4A1 are coinduced (Fig. 1c). The coregulation of microsomal cytochrome P450s from distinct families and peroxisomal acyl-CoA oxidase is in contrast to the differential dose–response of P450 2B1 and P450IIIAI to increasing doses of phenobarbital [19], which suggests differential regulation. Our data (Figs 1c and 2a, b) are consistent with a previous report, based on hepatocyte culture, suggesting that lauric acid

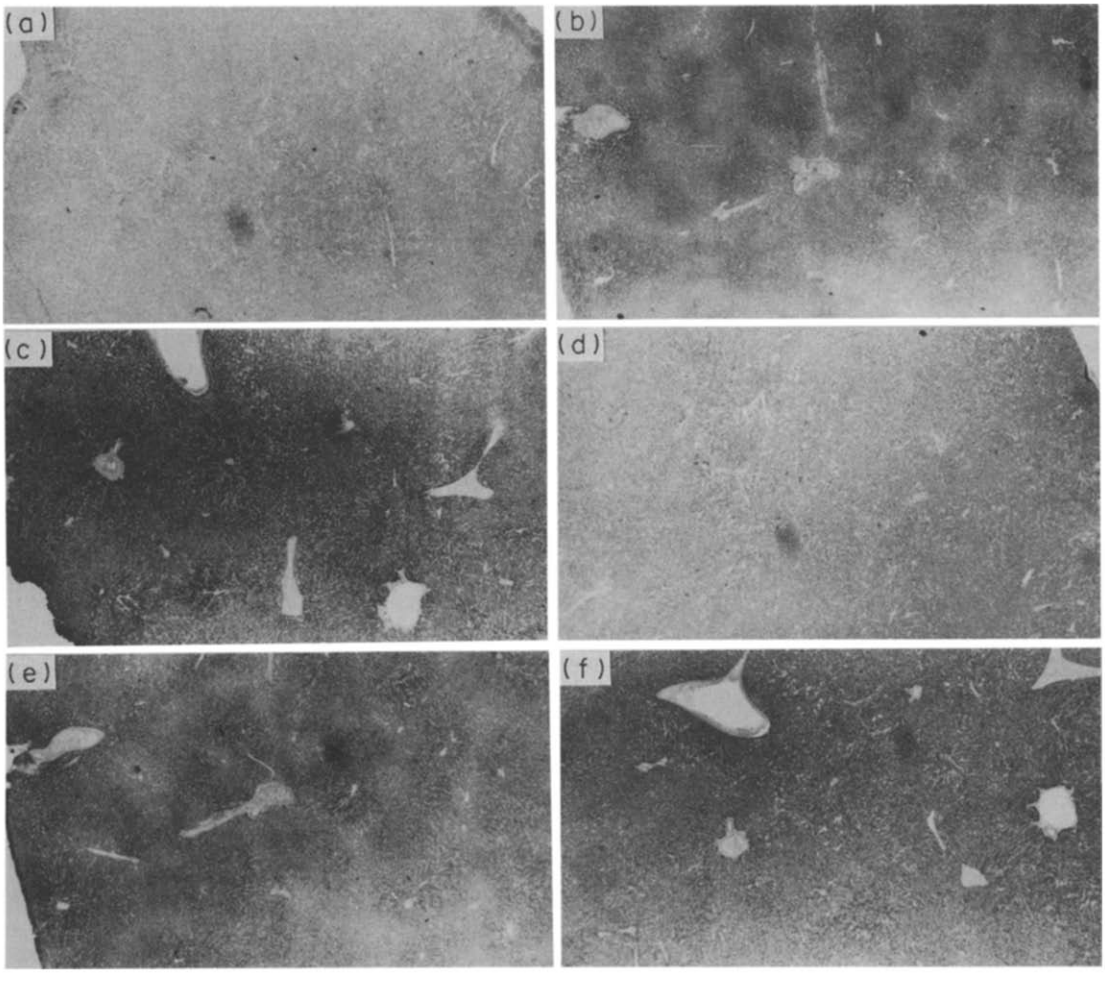

Fig. 4. Immunohistochemical localization of (a–c) P450 4A and (d–f) BFE in rat liver. Rats were treated with vehicle (a, d), 50 (b, e) or 300 (c, f) mg/kg of clofibric acid for 4 days, and the livers removed, fixed and sectioned. Antibodies against P450 4A and BFE were used for immunohistochemical localization as described in Materials and Methods. Results are representative of those obtained with four animals per dose group.

hydroxylase (cytochrome P450 4A) and acyl-CoA oxidase are induced by clofibric acid through a common mechanism [12]. Although acyl-CoA oxidase and P450 4A1 RNAs are induced with differing kinetics *in vivo* [41], dose–response studies (this study) demonstrate similar EC_{50} values for induction of these two RNAs. The recent demonstration of differential induction kinetics for these two RNAs *in vitro* [22] suggests that the mechanism of induction needs further examination.

The peroxisomal enzymes of β-oxidation have been shown to be regulated in parallel [11]. Therefore, we followed the induction of microsomal and peroxisomal proteins by western blotting with antisera to P450 4A and BFE, respectively. This revealed a parallel response to that detected by RNAse protection methodology, suggesting that the regulation of peroxisome proliferation is a transcriptional process [7, 11]. We then used these antisera to stain hepatocytes exposed to clofibric acid, to determine if the cells responded in a uniform manner. This revealed a graded variation in intensity of staining between hepatocytes exposed to identical concentrations of inducer (Fig. 3). This graded variation in hepatocyte response is distinct from the stark heterogeneity of response in primary cultures of hepatocytes exposed to phenobarbital (P450 2B1) or dexamethasone (P450 3A) [15].

In order to investigate whether the graded heterogeneity in induction response seen in hepatocytes *in vitro* was reflected in hepatocytes in the liver, rats were dosed with clofibric acid. In liver sections from rats treated with 50 mg/kg of clofibric acid, staining for both the BFI and P450 4A proteins is strongly induced in acinar zones 2 and 3 (Fig. 4), and this staining pattern becomes panacinar at higher doses of clofibric acid (300 mg/kg). These *in vivo* results are consistent with a previous report using the peroxisome proliferator, methylclofenopate [14], and suggest that the differential inducibility of hepatocytes throughout the liver acinus is not unique to an individual peroxisome proliferator, but may be common to the process of peroxisome proliferation.

In conclusion, this study demonstrates that P450 2B1, P450 4A1 and acyl-CoA oxidase RNAs are highly inducible in primary cultures of rat hepatocytes, and that these RNAs are coinduced with increasing doses of clofibric acid. P450 4A and BFE proteins are induced in parallel to the RNA induction response, but immunocytochemical examination of the cells revealed a graded variation in the induction response of individual hepatocytes. Dose–response studies with clofibric acid *in vivo* revealed the presence of a discrete hepatocyte population (zones 2 and 3 of the acinus) differentially responsive to induction of both P450 4A and BFE proteins.

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