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TRANSLACTATIONAL INDUCTION OF CYP4A EXPRESSION IN 10.5-DAY NEONATAL RATS BY THE HYPOLIPIDEMIC DRUG CLOFIBRATE

ANNEMARIE E. C. M. SIMPSON*‖, WILLIAM J. BRAMMAR†, MARGARET K. PRATTEN‡ and CLIFFORD R. ELCOMBE§

*Biology Research, R3, Knoll Pharmaceuticals, Pennyfoot Street, Nottingham, NG2 3AA; †Department of Biochemistry, Adrian Building, University of Leicester, University Road, Leicester, LE1 7RH; ‡Department of Human Morphology, Queen's Medical Centre, Nottingham, NG7 2UH; and §Zeneca Pharmaceuticals plc., Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ U.K.

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Abstract—Lactating mothers of 7.5-day neonatal rats were injected intraperitoneally with 500 mg kg^{-1} clofibrate for 3 consecutive days at 24-hour intervals; 24 hours after the final injection, the maternal cytochrome P450 4A (CYP4A) mRNA levels had risen 14- and 2.5-fold above the constitutive levels of expression seen in the liver and kidney, respectively. Lactational transfer of clofibrate to the suckling 10.5-day litter was demonstrated by the 15- and 5-fold elevation observed in the neonatal hepatic and renal CYP4A mRNAs, respectively, following suckling from drug-induced mothers. A significant decrease in the relative liver weights of these neonatal pups was seen following clofibrate exposure via maternal milk, in total contrast to the normally observed increase in liver/body weight ratios of rats treated with clofibrate. Western blot analysis using a polyclonal goat anti-rat CYP4A1 antibody also demonstrated a rise in the CYP4A protein levels in both the mothers and their litters following maternal clofibrate treatment.

Key words: CYP4A; clofibrate; lactational transfer; liver; fatty acid oxidation; arachidonic acid

Cytochrome P450s¶ (P450s) constitute a complex superfamily of proteins involved in the metabolism of both endogenous and exogenous compounds. The P450s, on one hand, are involved in the detoxification of certain chemicals, converting hydrophobic species to more hydrophilic derivatives that can subsequently be eliminated from the body; they can also, however, activate other substrates into toxic metabolites.

By December 1992, 221 genes and 12 putative pseudogenes had been characterised as belonging to the P450 superfamily [1]. This superfamily, which has existed for more than 3.5 billion years, consists of 36 gene families, members of which are found in both eukaryotes and prokaryotes. The CYP4 gene family is one of the oldest of the P450 families, having evolved about 1.25 billion years ago, just after the formation of the steroid biosynthetic genes. This family contains 6 subfamilies, among which the cytochrome P450 4A (CYP4A) subfamily encode proteins involved in lipid metabolism [2–5].

The CYP4A subfamily encodes several cytochrome P450 enzymes that are capable of hydroxylating the terminal ω-carbon and, to a lesser extent, the (ω-1) position of saturated and unsaturated fatty acids. The product of the CYP4A1 gene is a laurate ω-hydroxylase, which hydroxylates laurate at positions 11 and 12 with preferential hydroxylation at position 12 [6, 7]. Palmitate and arachidonate also serve as excellent substrates for this enzyme, which can also hydroxylate prostaglandins and leukotrienes to a lesser extent [8]. These latter substrates are more efficiently hydroxylated by the CYP4A4 gene product [9]. The CYP4A1, CYP4A2, and CYP4A3 genes are constitutively expressed in the rat, but the level of expression rises following administration of the hypolipidemic drug clofibrate [2, 4–6].

Although CYP4A1-catalysed hydroxylation of fatty acids at the terminal carbon has tended to be considered a catabolic process, recent investigations have suggested an alternative role for these reactions. Indeed, one of the reasons for the extensive interest in the CYP4A subfamily is related to the pharmacological activity of CYP4A-generated metabolites [10], particularly ω-hydroxyarachidonic acid, although which member of the CYP4A family is involved in the conversion of arachidonate to these signal metabolites is presently unknown.

Translactational transport of biologically active substances is important in mammalian physiology: passive immunity is acquired by this route [11, 12] because the neonatal animal obtains immunoglobulin G antibodies in the colostrum during suckling. Various chemical contaminants can also be found in human breast milk [13] that could have adverse effects on nursing infants. Borlakoglu *et al.* [14, 15] have recently demonstrated that CYP4A1 protein activity can be induced in neonates *via* lactational transfer of polychlorinated biphenyls.

Induction of peroxisome proliferation and peroxisomal β-oxidation enzymes, a phenomenon closely linked to clofibrate-induced CYP4A expression, has been observed in the livers of neonates suckling from hypolipidemic drug-induced mothers [16]. This study was carried out to determine whether or not maternally admin-

‖ Corresponding author. Tel. 0115 949 2538; FAX 0115 949 2330.

¶ Abbreviations: CYP,P450, cytochrome P450; DAB, 3, 3′-diaminobenzidine tetrahydrochloride; NaAc, sodium acetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; SET, 250 mM sucrose containing 5.4 mM ethylene diamine tetra-acetic acid and 20 mM Tris-(hydroxymethyl)-methylamine; SSPE, 3M sodium chloride, 20 mM ethylene diamine tetra-acetic acid and 200 mM sodium dihydrogen phosphate.

istered clofibrate was capable of modulating neonatal CYP4A expression *via* the mother's milk.

MATERIALS AND METHODS

Materials

Clofibrate (2, 4-(chlorophenoxy)-2-methylpropionate) was obtained from Sigma Chemical Co. (Poole, Dorset); [α-^{32}P]dCTP (3000Cimmol^{-1}) was from Amersham International (Amersham, U.K.). The 682 bp 3′ CYP4A1 cDNA probe was a probe derived from a 2.1 kb 3′ cDNA fragment of the CYP4A1 cDNA clone of Earnshaw *et al.* [3] kindly supplied by D. R. Bell (Zeneca Pharamceuticals Plc., Central Toxicology Laboratory, Alderley Park, Cheshire, U.K.). The large fragment of *E. coli* DNA polymerase I (Klenow fragment) was purchased from Bethesda Research Laboratories (Gibco BRL, Middlesex, U.K.). The polyclonal goat anti-rat CYP4A1 antisera were kindly provided by Zeneca Pharmaceuticals plc. This antibody was known to strongly react with the CYP4A1 protein, but also had some cross-reactivity with the CYP4A2 and CYP4A3 proteins. The CYP4A1 standard and methyl clofenapate-induced rat microsomal protein was kindly provided by Zeneca Pharmaceuticals Plc. All other chemicals were obtained from commercial sources, unless otherwise stated, and were of the highest purity available.

Animals, animal dosing and tissue processing

Wistar rats *(Rattus norvegicus)* obtained from the University of Leicester breeding colony were kept at a constant temperature of 20°C, humidity of 60%, and in an artificial 12 hr light/dark cycle. Diet RM1 or RM3 (Special Diet Services, Witham, Essex) and water were available *ad libitum.*

Lactating mothers were dosed intraperitoneally with 500 mg kg^{-1} of clofibrate in 500 μL of corn oil for varying time intervals, as indicated in the figure legends; all the mothers and their litters being culled, by terminal ether anaesthesia, when the litters were 10.5 days old. Control animals were given a similar injection of 500 μL of corn oil and were culled 24 hr later.

The maternal and neonatal tissues were removed immediately after death, snap-frozen in liquid nitrogen and stored at −70°C. All neonatal tissues were pooled. Maternal tissues were taken as internal controls to check that the drug was inducing CYP4A expression in the adult.

For the dot blot studies, two mothers and their litters were used for each time point. Four lactating mothers were dosed with clofibrate and four control mothers were dosed with corn oil; tissues from these animals were used in the Northern and Western blot analyses.

Construction of the pIV2S plasmid

The pIV2 construct was a gift from Dr. D. R. Bell (Zeneca Pharmaceuticals plc.) A full length CYP4A1 cDNA had been cloned into pUC19 [3] and a 1192 bp fragment (residues 965–2157) was excised by digestion with the restriction endonucleases *TaqI* and *EcoRI.* This 3′ cDNA fragment had then been subcloned into the *ClaI* and *EcoRI* restriction sites of the riboprobe gemini system pGEM^{R}-7Zf(+) vector (Promega), to give the pIV2 construct. Cleavage of this construct with the restriction endonuclease *SstI* released a 682 bp fragment that lacked the poly-A tail present in the original cDNA. This was subcloned into the phagemid pBluescript SK(+), according to Sambrook *et al.* [17]. The resulting construct was known as pIV2S.

Production of the probe for 18S ribosomal RNA

A probe specific for rat 18S rRNA was employed as a control to verify RNA loading and integrity. Two oligonucleotide primers, RR1: 5′-CATTCGAACGTCTGCCCTATCAAC-3′ and RR2: 5′-CCTCTTAATCATGGCCTCAGTTCC-3′, which flanked a 534 bp region of the 18S rDNA sequence, were synthesised on an Applied Biosystems 380B oligonucleotide synthesiser using phosphoramidite chemistry. The oligonucleotides were ethanol-precipitated and resuspended in water to a concentration of 78 ng μL^{-1} (10 μM).

The PCR reactions (50 μL total volume) were carried out in 1 × Parr Buffer (50 mM KCl/1.5 mM $MgCl_2$/0.1 mg mL^{-1} gelatin/10 mM Tris.HCl pH 8.0; Cambridge Bioscience), using 2 mM 'Ultrapure' dNTPS (Pharmacia), 1 mM primers (RR1 and RR2), 2.5 units *Taq* Polymerase (Cambridge Bioscience), and 0.5 μg of adult rat total genomic DNA.

25 cycles were carried out using the following touchdown temperature profile: denaturation; 96°C for 1 min; primer annealing; 70–50°C, at 2°C intervals for 1 min each; primer extension; 72°C for 5 min.

The DNA was recovered by phenol extraction and ethanol precipitation, and the amplified fragment (the 18S probe) was purified by low-melting-point agarose gel electrophoresis as described by Sambrook *et al.* [17]. 10 ng were radiolabelled to high specific activity using random primers, [α-^{32}P]dCTP and the Klenow fragment of *E. coli* DNA polymerase I according to the protocol of Sambrook *et al.* [17].

Preparation of total RNA

The tissue was homogenised in 10 mL g^{-1} lysis buffer (6M Urea/3 M LiCl/10 mM NaAc pH 5.2) along with 100 μL of 10% SDS per 10 mL of buffer. The homogenate was transferred to a siliconised Corex™ tube and stored at 4°C overnight.

The tubes were centrifuged at 10,000 rpm for 30 min at 4°C and the RNA pellet was resuspended in 5 mL of 6 M Urea/3 M LiCl and re-centrifuged. The pellet was gently resuspended in 10 mL of 10 mM Tris.HCl pH 7.5/0.5% SDS, a phenol extraction was carried out, and the RNA ethanol precipitated.

The RNA was resuspended in sterile diethylpyrocarbonate-treated water, the concentration was determined by spectrophotometry at 260 nm, and the solution was stored at −70°C. Before use, the integrity of the RNA samples was checked by electrophoresis through a 1% agarose gel. Visualisation of the 28S and 18S rRNA species as discrete bands was taken to indicate that the RNA samples were largely intact.

Dot blot analysis

Dot blot analysis was carried out in duplicate, according to Sambrook *et al.* [17], using a nylon Pall Biodyne-A™ transfer membrane and RNA loadings ranging from 125 ng to 5 μg. Whole brain RNA taken from con-

trol adult females was used as a negative control. The RNA was cross-linked to the membrane by exposure to UV light. The membranes were hybridised with a [α-^{32}P]dCTP-radiolabelled CYP4A1 cDNA probe with a specific activity of 1.8×10^9 dpm μg^{-1}. The CYP4A1 cDNA probe was radiolabelled using random primers, [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I as described by Sambrook *et al.* [17].

Northern blot analysis

The RNA species (30 μg per track) were separated in a 1.25% agarose, 17.4% formaldehyde gel according to the protocol of Sambrook *et al.* [17]. The RNA species were transferred to a nylon Pall Biodyne-A™ transfer membrane *via* capillary action and cross-linked via UV irradiation. The marker track was removed from the rest of the membrane and the RNA was visualised by staining the track in 0.5 M NaAc pH 5.2 containing 0.04% methylene blue for 15 min.

Membrane hybridisation

The membranes were prehybridised at 42–45°C in 5 × SSPE/50% formamide/5 × Denhardt's solution/0.5% SDS and denatured 50 μg mL^{-1} Herring sperm DNA, for 30 to 60 min. Hybridisation was carried out overnight in fresh hybridisation buffer at 42–45°C in a shaking water bath with Herring sperm DNA as a blocking agent and 0.4 ng μL^{-1} of a heat denatured [α-^{32}P]dCTP random primed CYP4A1 cDNA probe.

At the end of the hybridisation period, the membranes were washed 3 times for 20 min each in 2 × SSPE/0.1% SDS at 42–45°C, twice for 20 min in 0.5 × SSPE/0.1% SDS at 65°C and, finally, 2 times at 65°C for 20 min each, in 0.1 × SSPE/0.1% SDS. The membranes were exposed to Fuji RX™ film, along with intensification screens, for varying periods of time.

Quantitation of mRNA levels

After exposure of the probed membranes to X-ray photographic film, the signal intensities were semiquantitated *via* an ultroscan 2202 laser densitometer linked to an Apple II computer. Linear regression analysis was carried out on the data and those data in the linear-response range of the film were analysed.

Protein concentration determination

Protein concentrations were determined using the Pierce BCA™ assay [18], with bovine serum albumin as a standard. The absorbance was read at 562 nm. A standard calibration curve was produced using BSA diluted in SET buffer pH 7.4.

Western blot analysis of proteins

The tissues were homogenised in 3 volumes of SET buffer pH 7.4, centrifuged for 10 min at 2000 rpm at 4°C, and the supernatants (crude homogenates) either assayed immediately or stored at –70°C. The proteins in the homogenates were dissociated into their constituent polypeptides via SDS-PAGE using a 7.5% resolving gel and 5% stacking gel. 60 μg of samples were loaded along with 1 pmole of the CYP4A1 standard and Bio-Rad broad range SDS-PAGE molecular weight standards. 8 μg of microsomal protein prepared from rats treated with the peroxisome proliferator, methylclofenapate (25 mg kg^{-1} in corn oil, intraperitoneal injections for 4 days), and culled 24 hours after final dose (supplied by S. Watson, Zeneca Pharmaceuticals plc.) were used as a positive control.

After electrophoresis, the gel was soaked in 25 mM Tris/190 mM glycine/4% methanol buffer for about 5 min and the proteins transferred to a nylon sheet of Pall Biodyne-A™ *via* electroblotting overnight. The nylon sheet was then washed twice, for 10 min each, in Tris buffer pH 8.2, blocked for an hour in 5% BSA-Tris at 37°C, and then washed twice, for 10 min each, in Tris pH 8.2 at room temperature. It was incubated for 2.5 hr at room temperature with the primary antibody, using a 1:100 dilution made up in 0.1% BSA-Tris and 1% (v/v) normal swine serum (Dakopatts). The sheet was washed 4 times, for 15 min each, in 0.1% BSA-Tris at room temperature and then incubated with a 1:100 dilution of the secondary antibody, peroxidase-labelled rabbit anti-goat IgG (ICN Biomedicals Inc.) diluted in 0.1% BSA-Tris, for an hour at room temperature. The sheet was subsequently washed 4 times, for 15 min each, in 0.1% BSA-Tris at room temperature.

30 mg of 4-chloro-1-naphthol (Sigma) were added to 10 mL of methanol and then 50 mL of TBS pH 7.9 were added to this. Immediately prior to addition to the filter, 30 μL of 30% (w/w) hydrogen peroxide (Sigma) were added to the 4-chloro-1-naphthol solution. After the bands had developed, the filter was transferred to several changes of water to stop the reaction, dried on filter paper, and stored in the dark.

RESULTS

The CYP4A subfamily is highly complex, consisting of 13 genes, to date, of which four are expressed in the rat: the CYP4A1, CYP4A2, CYP4A3, and CYP4A8 genes. The CYP4A1, CYP4A2, and CYP4A3 genes are all expressed in the liver and also show a high degree of similarity both at the amino acid and nucleotide level [4, 5], although there is a low sequence similarity of the CYP4A2 and CYP4A3 sequences with bases 1 to 39 of the CYP4A1 gene. The 3′-sequence (exon 8–exon 13) of the CYP4A1 transcript [the region corresponding to the cDNA probe] had more than 80% sequence identity with the CYP4A2 and CYP4A3 transcripts in this region. Thus, Northern blot analysis with the CYP4A1 cDNA probe was likely to detect not only the CYP4A1 mRNA but also those of the other subfamily members, if they were present. The messenger species detected by the CYP4A1 probe are, therefore, referred to as the CYP4A mRNAs.

Induction profile of liver CYP4A mRNAs

A time-course experiment was carried out to see whether CYP4A expression could be induced translactationally and, if so, the dot blot would reveal the dosing regimen to use to obtain maximal induction. The membranes were stripped of the CYP4A1 cDNA probe and rehybridised with the 18S probe, with a specific activity of 1.4×10^9 dpm μg^{-1}, to allow normalisation of the RNA loadings.

Figures 1 and 4a show that, in the maternal liver, there was a large increase in the intensity of the signal 24 hr after drug administration. This increase in the level of the CYP4A messages was approximately 7-fold and with repeated dosing it rose even further, so that with 3 injections of clofibrate at 24-hr intervals, 24 hours after the final injection the level of CYP4A messages was about 14-fold higher than that seen in the control moth-

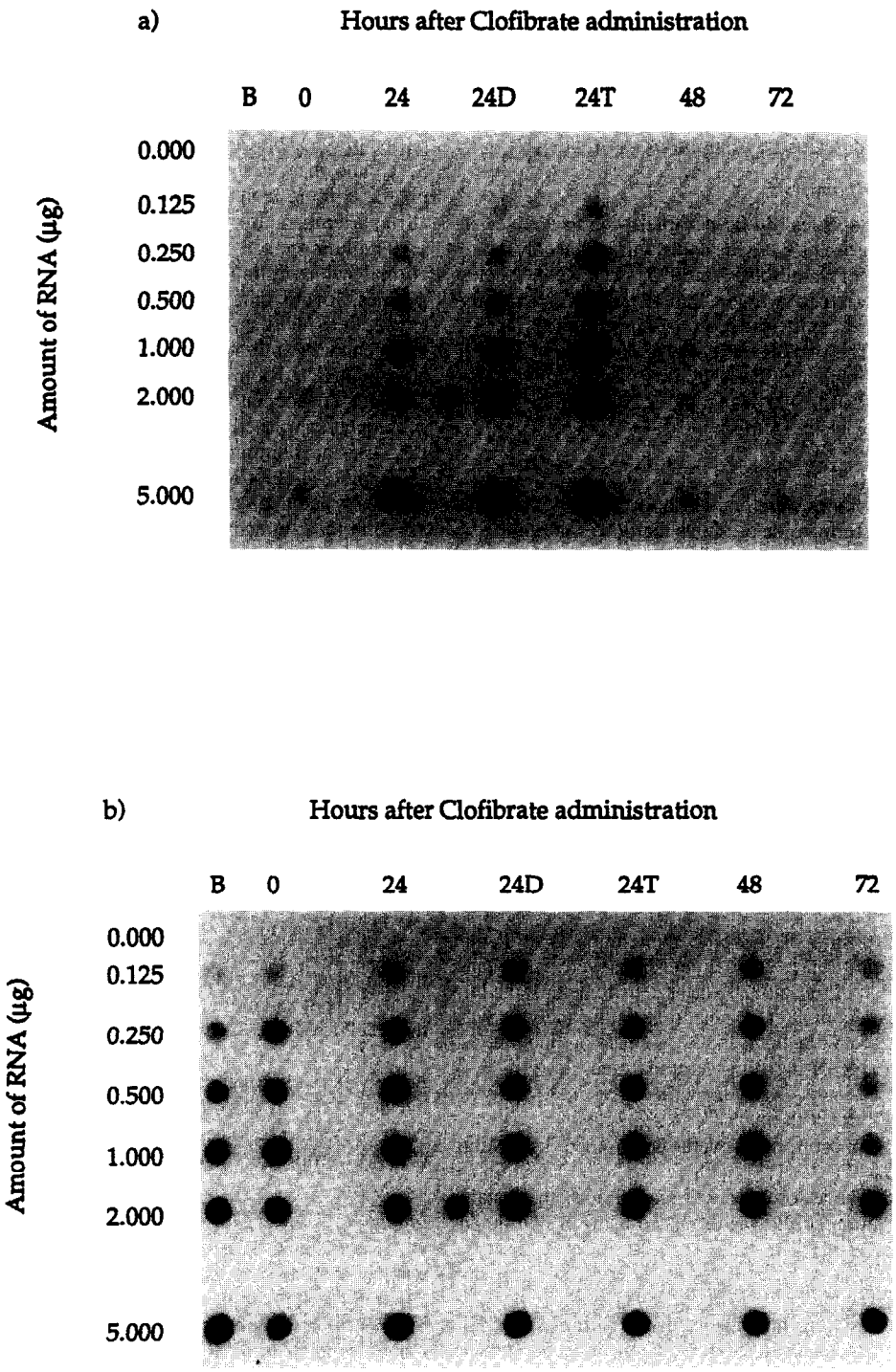

Fig. 1. Dot blot analysis of maternal liver RNA of 10.5-day neonates. (a) Maternal rats of 10.5-day neonatal litters were given an intraperitoneal injection of clofibrate (500 mg kg^{-1}) and killed at various time intervals after drug administration: 24, 48, and 72 hr. Females (24D) were given 2 doses of clofibrate, 24 hr apart and were killed 24 hr after the last injection. Females (24T) were dosed 3 times, 24 hr apart and were killed 24 hr after the last dose. The control mothers (0) were injected with corn oil and killed 24 hr later. Whole brain RNA (B) from noninduced female was used as a negative control. RNA was applied to a Pall Biodyne™-A membrane and probed with a random primed [α-^{32}P]dCTP-radiolabelled CYP4A1 cDNA probe. The membrane was exposed to X-ray film for 7 weeks at –70°C. (b) The CYP4A1 cDNA probe was removed from the membrane and, to normalise the amount of RNA loaded, the membrane was subsequently rehybridised with the 18S probe. The membrane was exposed to X-ray film for 1 hr at room temperature.

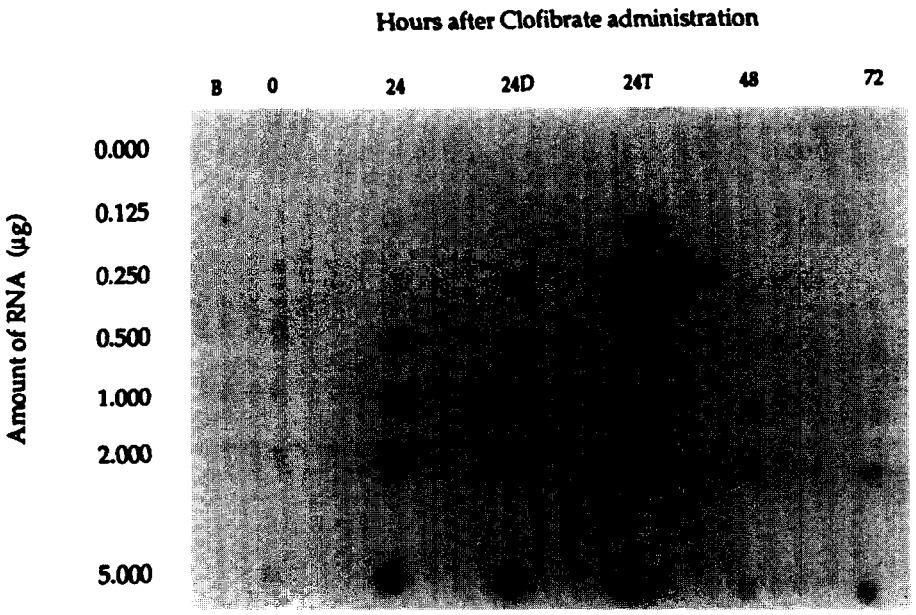

Fig. 2. Dot blot analysis of liver RNA from 10.5-day neonates suckling from drug-induced mothers. 10.5-day neonatal litters that were suckling from drug-induced mothers (500 mg kg^{-1} clofibrate) were killed at various time intervals after maternal drug administration: 24, 48, and 72 hr. Mothers (24D) were given 2 doses of clofibrate, 24 hr apart and their litters killed 24 hr after the last injection. Mothers (24T) were dosed 3 times, 24 hr apart and both the mothers and their litters were killed 24 hr after the last dose. The control mothers (0) were injected with corn oil and their litters killed 24 hr later. Whole brain RNA (B) from noninduced adult females was used as a negative control. The RNA was applied to a Pall Biodyne-A™ membrane and probed with a random-primed [α-^{32}P]dCTP-radiolabelled CYP4A1 cDNA probe. The membrane was exposed to X-ray film for 7 weeks at −70°C.

ers. There was a dramatic decrease in the level of the messages 48 and 72 hr after dosing the mother with the inducer, suggesting a short half-life of the messages.

This pattern of induction was also mirrored in the suckling 10.5-day neonatal livers (Figs. 2 and 4b). The level of induction seen in those neonates suckling from mothers who had been injected 24 hr previously was 6-fold and, as seen in the maternal liver, the level of the messages continued to rise with repeated dosing of the nursing mothers. Maximal induction in the neonatal liver was achieved by injecting the nursing mothers 3 times and taking the tissues 24 hr after the third injection; the level of the CYP4A messages at this time being approximately 13-fold above that seen in the noninduced animals.

The dot blot, therefore, demonstrated that the drug clofibrate could be transported from a mother to her suckling pups, via her milk, and cause induction of the CYP4A messages in neonatal tissues.

Induction profile of renal CYP4A mRNAs

The levels of the CYP4A mRNAs in the maternal and neonatal kidney (Figs. 3 and 4) were lower than those seen in the respective livers. The CYP4A mRNAs in the kidney followed the same induction profile as that seen in the liver, maximal expression being seen after 3 consecutive maternal injections, 24 hr apart, and the kidneys being removed 24 hr after the third clofibrate injection.

A very low level of basal expression was detectable in the kidneys of the control nursing mothers and their litters; it had nearly doubled 24 hr after a single injection of clofibrate. With 3 injections of the drug, the level of CYP4A mRNA expression had risen nearly 2.5 × and 5 × above the constitutive expression level in the mothers and their suckling neonates, respectively. By 48 and 72 hr after a single dose of clofibrate, the level of CYP4A expression had decreased, approaching the constitutive level.

The effect of lactational transfer of clofibrate on liver size in the 10.5-day neonates

The relative liver weights of the suckling neonates from the different groups used in the dot-blot analysis were investigated. Immediately following their deaths, the pups were weighed, their livers removed, and these also weighed. The liver weights were then expressed as liver/body weight ratios and a two-tailed Mann-Whitney U-test performed on the data (Table 1).

Statistical analysis of the data revealed that translactational exposure to clofibrate caused a significant decrease in the relative liver weights of those pups that were culled 24 hr after the final maternal injection of clofibrate. These results were in contrast to those of a similar investigation carried out on 24-day neonates, in which an increase in the relative liver weight was noted 24 hr following an intraperitoneal injection of clofibrate (500 mg kg^{-1} [19]).

Hepatomegaly, as indicated by an increase in the liver/body weight ratios of animals treated with clofibrate, is a widely accepted phenomenon [20–23]. However, here it appeared that clofibrate received *via* maternal milk had not caused the normally observed increase in liver size, but had had the opposite effect. In addition, the pups also tended to be smaller, their body weights as well as their

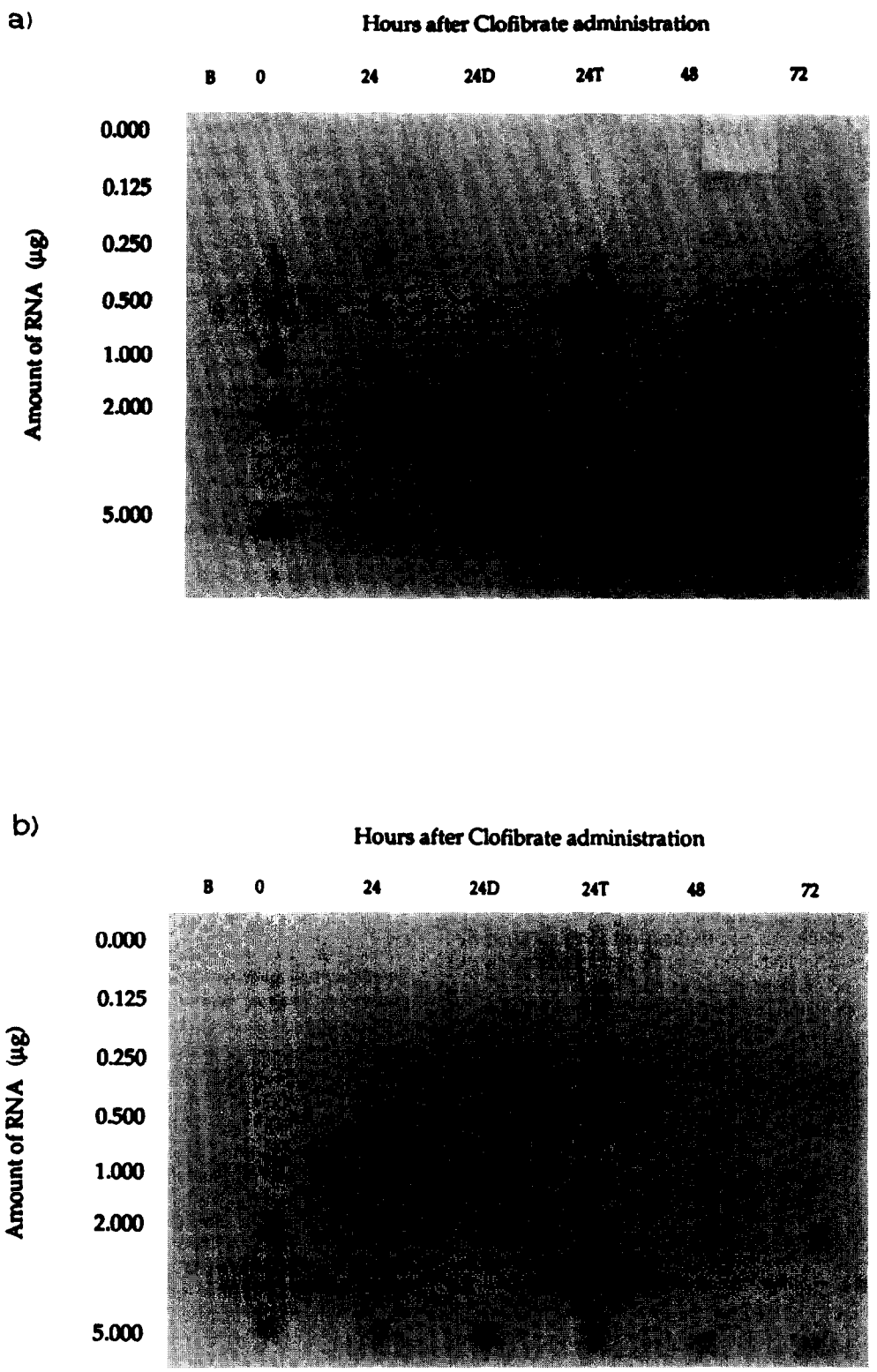

Fig. 3. Dot blot analysis of maternal and 10.5-day neonatal kidney RNA. Maternal rats of 10.5-day neonates were given an intraperitoneal injection of clofibrate (500 mg kg^{-1}) and both the mothers and their litters were killed at various time intervals after maternal drug administration: 24, 48, and 72 hr. Females (24D) were given 2 doses of clofibrate, 24 hr apart and both the mothers and their litters were killed 24 hr after the last injection. Females (24T) were dosed 3 times, 24 hr apart and both the mothers and litters were killed 24 hr after the last dose. The control mothers (0) were injected with corn oil and both the mothers and litters killed 24 hr later. Whole brain RNA (B) from a noninduced adult female was used as a negative control. Maternal RNA (panel A) and neonatal RNA (panel B) were applied to Pall Biodyne-A™ membranes and probed with a random-primed [α-^{32}P]dCTP-radiolabelled CYP4A1 cDNA probe. The membrane were exposed to X-ray film for 7 weeks at –70°C.

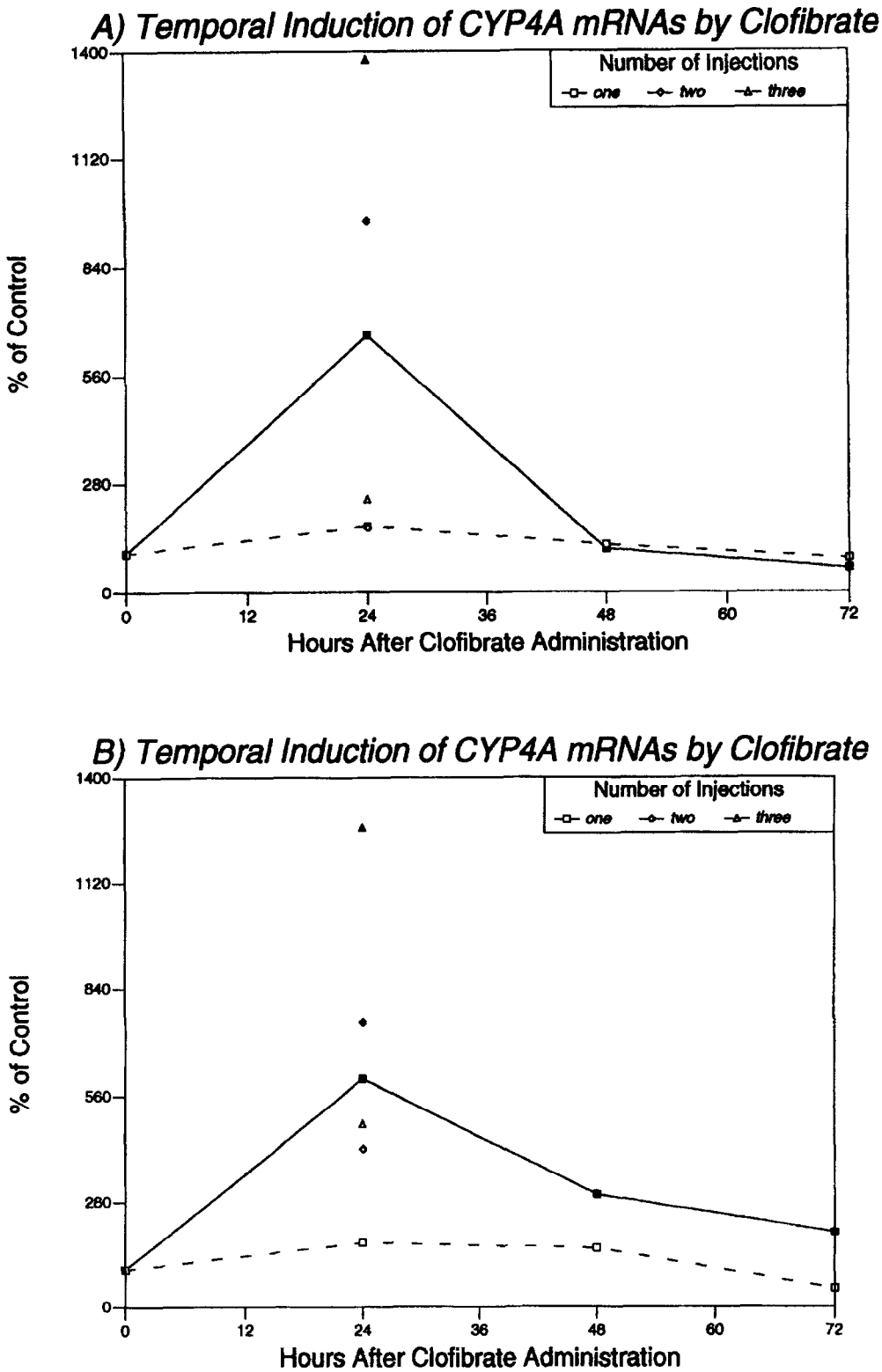

Fig. 4. Induction profile of CYP4A mRNAs after clofibrate administration. Dot blot analysis was carried out using 500 ng of total RNA, or 1 μg in the case of maternal kidney. The RNA was extracted from liver or kidney of nursing mothers (Fig. 4a) and their 10.5-day neonatal litters (Fig. 4b), exposed to clofibrate (500 mg kg^{-1}) for varying periods of time. The RNA was applied to Pall Biodyne-A™ membranes and probed with a random-primed [α-^{32}P]dCTP-radiolabelled CYP4A1 cDNA probe. The dot blots were semiquantitated by scanning densitometry and the control values (zero time point) assigned an arbitrary value of 100%. The amount of RNA was normalised by hybridisation with the 18S probe. Each time point is the mean of 2 samples, these being pooled for the neonatal tissues. The liver values are represented by closed symbols and the kidney values are represented by open symbols.

Table 1. The effect of clofibrate on liver size in 10.5-day-old neonates suckling from drug-induced mothers

Treatment	Number of animals	Dose level (mg kg^{-1})	Number of injections	Time mother and litter culled after last maternal injection (hr)	Body weight (g)	Liver/body weight ratio (%)
Corn oil	22	—	1	24	19.14 ± 4.75	3.56 ± 0.77
Clofibrate	23	500	1	24	17.69 ± 1.72	2.76 ± 0.25**
Clofibrate	26	500	2	24	14.34 ± 2.53**	2.80 ± 0.18**
Clofibrate	23	500	3	24	13.11 ± 0.82**	2.95 ± 0.22*
Clofibrate	24	500	1	48	22.19 ± 4.24	3.21 ± 0.30
Clofibrate	22	500	1	72	17.83 ± 2.93	3.13 ± 0.46

The drug or vehicle was administered to the lactating mothers of the 10.5-day-old neonates by intraperitoneal injections, 24-hr intervals being left between consecutive injections. The values expressed for body weight and liver/body weight ratios are means ± SD. P values for results significantly differed (Mann-Whitney U test) from control data at $*P < 0.05$ and $**P < 0.01$.

liver weights being lower than those of the control group (Table 1). It could be that the clofibrate was somehow affecting the ability of the neonates to thrive. A histological examination of the induced and control neonatal liver sections revealed no obvious morphological differences between them. In an investigation carried out by Cibelli *et al.* [24], clofibrate treatment of pregnant rats at different gestational ages was shown to abolish the weight gain normally observed during pregnancy, and the liver weight of the animals remained unchanged with respect to the control values. In the fetuses, body weight was significantly lower than that of the control group toward the end of prenatal development and at birth.

Northern blot analysis of 10.5-day neonatal tissues

Maternal tissues of 10.5-day neonates. The Northern blot analysis revealed constitutive expression of CYP4A mRNAs in all the four lobes of the control liver, as shown by the presence of a message of about 2.4 kb (Fig. 5a). After clofibrate administration, there was a dramatic increase in the intensity of the signal, indicating an increase in the amount of message present. When quantified, this signal increase reflected roughly an 11-fold rise in the level of the messages above that seen in the uninduced rats. This is only an approximation because 30 μg of RNA were loaded in each track and, at this high loading level, the linear relationship between RNA and signal intensity was lost, as determined by linear regression analysis (data not shown). Constitutive expression of the CYP4A mRNAs was also seen in the control kidney (Fig. 5a): however, clofibrate administration did not cause such a dramatic increase in the mRNA levels as that demonstrated in the liver. Drug administration increased the level of the renal messages by about 1.3-fold.

10.5-day neonatal tissues. An extremely low level of basal CYP4A mRNA expression was demonstrated in the 10.5-day neonatal liver lobes (Fig. 5b). In fact, the signal intensities were so low that they were unresolvable when scanned by the laser densitometer although they were visible with the naked eye. Thus, although significant levels of CYP4A mRNA were detected after induction, the magnitude of the induction could not be determined.

The CYP4A messages were also expressed in the kidneys taken from those neonates suckling from control and drug-injected mothers, even though their levels of expression were barely detectable by Northern blot analysis. Although the Northern blot analysis did not show convincing renal induction, the dot blot (Fig. 3) and Western blot (Fig. 6) analyses clearly did.

Western blot analysis of control and clofibrate-induced maternal 10.5-day suckling neonatal tissues

Western blot analysis was carried out on homogenates of livers and kidneys from the control and experimental nursing mothers and their litters, to examine whether induction of CYP4A at the mRNA level resulted in an equivalent rise in the protein levels.

Maternal tissues. The Western blot revealed the presence of two bands with similar molecular weights of 51.5 and 52 kD (Fig. 6) in homogenates from both the liver and kidney. This was in agreement with previous Western blot analyses using this polyclonal antibody, which is known to produce two bands [2, 5] referred to as the "CYP4A" proteins. In the liver and kidney, the slower migrating band is believed to be the CYP4A3 protein [25]; the faster migrating species, in the male, is thought to consist of two unresolved proteins: CYP4A2 and CYP4A3 and, in the female, to be made up of only the CYP4A1 protein [26]. Thus, in this situation, the lower maternal band presumably contained only the CYP4A1 protein, this protein running with the same mobility as the known CYP4A1 standard (Fig. 6, track 9).

A dramatic increase in the intensity of both bands was evident when comparing control and induced tracks of both tissues, indicating an elevation of the protein levels between the 2 states. However, there seemed to be a preferential induction of the lower CYP4A1 band in the maternal liver, as had previously been reported in the adult rat [27, 28]. This was not apparent in the kidney where, on the contrary, the induction of the upper CYP4A3 band appeared to be more noticeable. The bands were more intense in the liver tracks compared to the kidney tracks, which correlated well with the levels of CYP4A mRNA detected *via* dot and Northern blot analysis.

10.5-Day neonatal tissues. The CYP4A immunoreactivity demonstrated in the control and drug-induced mothers was also present in the suckling 10.5-day neonatal homogenates. As with the mothers, the level of expression of the CYP4A proteins, as indicated by the intensity of the staining of the bands, was greater in the liver than in the kidney. In the liver homogenate prepared from neonates suckling from control mothers (Fig. 6, track 5), the band corresponding to the CYP4A3 protein was marginally more intense than the lower band. However, in the clofibrate-induced homogenates (Fig. 6,

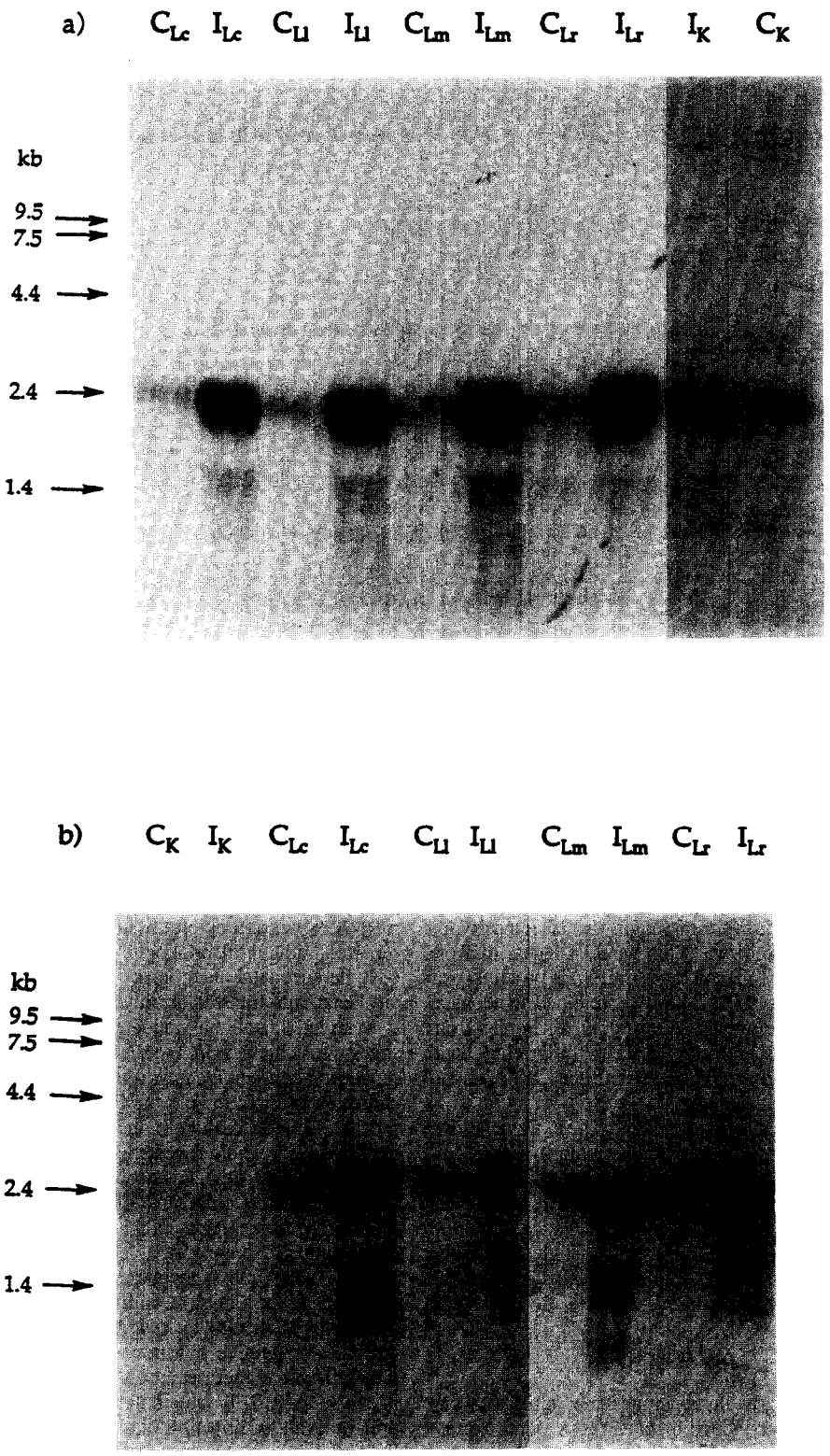

Fig. 5. Induction of CYP4A mRNAs in maternal and 10.5-day neonatal tissues. Northern blot analysis of total RNA (30 μg) isolated from either the mothers (Panel A) or their suckling 10.5-day neonates (Panel B) was carried out. The induced mothers (I) were treated with 500 mg kg^{-1} clofibrate and the control animals (C) were dosed with the vehicle, corn oil. Tissues taken were liver, right lobe (Lr), median lobe (Lm), left lobe (Ll), and caudate lobe (Lc) and kidney (K). The blots were probe with a CYP4A1 cDNA probe as described under Materials and Methods and were exposed to X-ray film for 3 weeks at −70°C. A BRL RNA marker was used and the sizes are indicated.

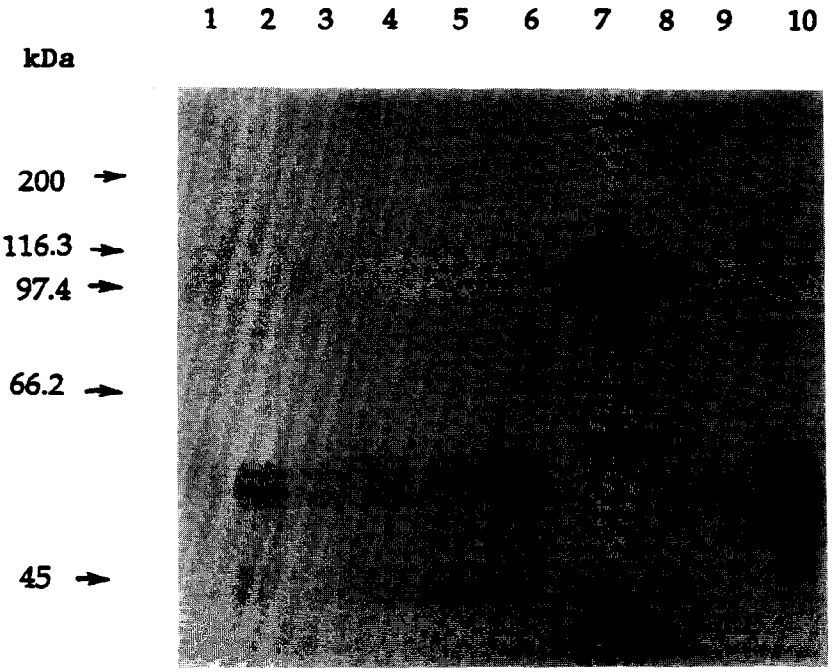

Fig. 6. Immunodetection of CYP4A proteins in crude rat homogenates from control and clofibrate-treated nursing mothers and their suckling 10.5-day neonates, using a polyclonal anti-CYP4A1 antibody. 60 μg of crude homogenate proteins from drug-treated or control mothers and their 10.5-day suckling neonatal rats were resolved by electrophoresis in a 7.5% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose. The immunoblot was carried out with a polyclonal goat anti-rat CYP4A1 antibody (diluted 1:100) followed by peroxidase-linked rabbit anti-goat IgG (diluted 1:100). The maternal rats were given 3 intraperitoneal injections of clofibrate (500 mg kg^{-1}), at 24 hr intervals or corn oil as a single injection 24 hr prior to death. 1. Control maternal liver; 2. Induced maternal liver; 3. Control maternal kidney; 4. Induced maternal kidney; 5. Control neonatal liver; 6. Induced neonatal liver; 7. Control neonatal kidney; 8. Induced neonatal kidney; 9. 1 pmole of CYP4A1 standard; 10. 8 μg of methylclofenapate-induced rat microsomal protein. The Biorad SDS-PAGE molecular weight markers were myosin (200,000); β-galactosidase (116,250): phosphorylase b (97,400); BSA (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); trypsin inhibitor (21,500); lysozyme (14,400); and aprotinin (6,500).

track 6) both bands were of roughly equal intensity, as judged by the naked eye. This would suggest that the induction of the CYP4A1 and CYP4A2 proteins was slightly above that of the CYP4A3 protein. The neonatal homogenates were prepared from pooled samples from both sexes and, therefore, the faster migrating band probably contained a mixture of both the CYP4A1 and CYP4A2 proteins.

In the kidney, the levels appeared to be extremely low; the CYP4A3 protein comprised the majority of the immunoreactivity in both the control and induced states. The lower band was very faint, indeed, in the control homogenate and was induced by a very small amount in the induced homogenate, indicating a slight rise in the level of the CYP4A1 and CYP4A2 proteins between the control and induced condition.

DISCUSSION

This study clearly demonstrates that clofibrate can mediate lactational induction of CYP4A gene expression. This effect could clearly be due to translactational transfer of the drug or its metabolites, or to an indirect effect *via* drug-induced changes in maternal metabolism. Clofibrate is known to cause changes in the lipid profile and, thus, it is conceivable that the clofibrate-dependent change in the type and amount of lipids in the maternal milk could additionally contribute to CYP4A induction in the neonate. Translactational induction of CYP4A mRNA expression by clofibrate has not previously been reported, although Borlakoglu *et al.* [14, 15] have demonstrated neonatal induction of CYP4A1 protein activity *via* lactational transfer of polychlorinated biphenyls.

Maximal lactational induction of the CYP4A messages was achieved *via* 3 intraperitoneal injections of the mother on consecutive days and culling both the mother and litter 24 hr following the final dose. This induction profile differed from that in the adult female and 24-day neonates [19], where repeated doses of clofibrate at 24-hr intervals did not elevate the levels of the CYP4A mRNAs. This difference may be related to the possible modulation of receptive cells by hormonal changes during lactation or pregnancy.

The CYP4A messages were present in all 4 lobes of the liver and the kidney of both control and drug-induced neonates. As seen in the adult and 24-day neonates [19], both the constitutive and induced levels of liver CYP4A mRNAs were higher than those in the comparative states in the kidney. This situation was also observed at the protein level, as shown by Western blot analysis of crude liver and kidney homogenates. In the maternal liver, there appeared to be a preferential induction of the CYP4A1 protein and in the kidney the CYP4A3 protein was preferentially induced. The levels of protein expression in the neonates was very low, but still detectable, and an induction of the CYP4A proteins by the drug was apparent.

Regulation of the activity of the CYP4A enzyme sys-

tem during neonatal life could be important in the response to nutritional changes. In rapidly growing newborn pups, the requirements for essential fatty acids are particularly high and the neonatal suckling period is characterised by an increase in fatty acid availability, primarily from maternal milk. Of milk fats, 95% are in the form of triacylglycerols: rat milk triacylglycerols contain up to 40% of medium-chain fatty acids [29], which have been shown to induce CYP4A1 in cultured hepatocytes [30]. The activity of the CYP4A1 enzyme in the suckling neonates would aid in the metabolism of the large amounts of fats present in the mother's milk (10g/100g) [31], leading to subsequent β-oxidation of the long-chain dicarboxylic acids. Lockwood and Bailey [32] demonstrated that the ability to degrade fatty acids is greater during suckling, when a high fat diet is being consumed, than during fetal or postweaning stages of life, when the diet is predominantly carbohydrate. Developmental expression of other P450 genes have been reported, with CYP1A gene expression being closely related to the diet changes accompanying weaning [33].

Developmental changes in the peroxisomal fatty acid oxidation system have been shown in rat liver [34], with palmitoyl CoA oxidase activity rising rapidly immediately after birth. The CYP4A enzymes involved in lipid metabolism could work in synchrony with the peroxisomal enzymes, playing an important role in providing short- and medium-chain fatty acyl CoA and $NADPH_2$ for the mitochondria to produce the ATP required for the rapid phase of neonatal growth.

Hormonal regulation of CYP4A gene expression is established because the CYP4A2 message is absent in adult female liver and kidney but is present in those tissues of the adult male rat [26]. Hepatic CYP4A2 expression is suppressed by continuous exposure to high levels of growth hormone, characteristic of adult females. In the kidney, the CYP4A2 mRNA also displays male-specific expression but, here, it is modulated by a pathway involving testosterone as a positive regulator [26]. Such hormonal changes and their consequent effects on gene expression normally accompany the weaning period [31].

Although CYP4A1-catalysed hydroxylation of fatty acids at the terminal carbon has tended to be considered as a catabolic process, recent investigations have suggested an alternative role for these reactions. Arachidonic acid, a potential substrate for the CYP4A1 gene product, can be metabolised by one of three pathways, depending on the tissue and cofactor availability [35]. The P450-derived metabolites of arachidonic acid have a wide and contrasting spectrum of biological activities [reviewed by 10], some causing inhibition of Na^+-K^+ ATPase and dilation of blood vessels [36] and others stimulating Na^+-K^+ ATPase and being potent vasoconstrictors [37]. The biological properties of the products of arachidonic acid metabolism suggest that they are potentially important in the regulation of renal function and general circulation.

The arachidonic acid metabolites, possibly CYP4A-generated, may act initially as second messengers within the cell of origin and undergo transformation to products exhibiting vasoactivity within the local environment.

In conclusion, maternally administered clofibrate is capable of effecting induction of the CYP4A gene system in suckling neonates. The drug elevates the level of CYP4A mRNAs and proteins in both the liver and kidney, although the levels in the latter are lower than those in the liver. The precise physiological role(s) of the CYP4A proteins is unknown but these proteins would allow the pups to metabolise the fatty acids that would be present at high concentrations in maternal milk.

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