Induction of the multispecific organic anion transporter (*cMoat/mrp2*) gene and biliary glutathione secretion by the herbicide 2,4,5-trichlorophenoxyacetic acid in the mouse liver

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The canalicular multispecific organic anion transporter, cMoat, is an ATP-binding-cassette protein expressed in the canalicular domain of hepatocytes. In addition to the transport of endo- and xenobiotics, cMoat has also been proposed to transport GSH into bile, the major driving force of bile-acid-independent bile flow. We have shown previously that the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), a peroxisome-proliferator agent, significantly increases bile-acid-independent bile flow in mice. On this basis, the effect of the herbicide on *cMoat* gene expression was studied. A 3.6-fold increase in *cMoat* mRNA levels and a 2.5-fold increase in *cMoat* protein content were observed in the liver of mice fed on a diet supplemented with 0.125 % 2,4,5-T. These effects were due to an increased rate of gene transcription (3.9-fold) and were not associated with peroxisome proliferation. Significant increases in bile flow

(2.23 ± 0.39 versus 1.13 ± 0.15 $\mu\text{l}/\text{min}$ per g of liver; $P < 0.05$) and biliary GSH output (7.40 ± 3.30 versus 2.65 ± 0.34 nmol/min per g of liver; $P < 0.05$) were observed in treated animals. The hepatocellular concentration of total glutathione also increased in hepatocytes of treated mice (10.95 ± 0.84 versus 5.12 ± 0.47 mM; $P < 0.05$), because of the induction (2.4-fold) of the heavy subunit of the γ -glutamylcysteine synthetase (*GCS-HS*) gene. This is the first model of co-induction of *cMoat* and *GCS-HS* genes *in vivo* in the mouse liver, associated with increased glutathione synthesis and biliary glutathione output. Our observations are consistent with the hypothesis that the cMoat transporter plays a crucial role in the secretion of biliary GSH.

Key words: ABC transporter, bile flow, canalicular transport, γ -glutamylcysteine synthetase heavy-subunit gene, xenobiotics.

INTRODUCTION

Transport across the hepatocyte canalicular membrane into the bile is a critical step in the elimination of endogenous and exogenous compounds in mammals [1,2]. Several enzymes catalyse the conversion of these compounds into amphiphilic anionic conjugates with glutathione, glucuronate or sulphate [1,2]. Excretion of these conjugates into bile is mediated by an ATP-dependent export pump called cMoat (canalicular multispecific organic anion transporter, or *mrp2*), which has been cloned recently [3–6]. This transporter is a transmembrane glycoprotein of 190 kDa that is normally expressed in the canalicular domain of hepatocytes, belonging to the ATP-binding-cassette transporter family, and encoded by the *cMoat* gene in rodents and the *cMOAT* gene in humans [7]. The *cMoat* gene is not expressed in the liver of the mutant rat strain Groningen yellow/transport-deficient (GY/TR[−] or TR[−]) [3,4], which is deficient in the biliary transport of glutathione conjugates, leukotriene C₄, bilirubin glucuronide and other amphiphilic anions [8,9]. In TR[−] rats the *cMoat* gene was found to bear a single nucleotide deletion resulting in a stop codon and absence of the cMoat protein [4,6]. Humans who have an autosomal-recessive liver disorder characterized by chronic conjugated hyperbilirubinaemia and impaired hepatobiliary transport of non-bile-salt organic anions, called Dubin–Johnson syndrome, share a similar phenotype to the GY/TR[−] mutant rats [10,11]. Interestingly, the molecular mechanism of this rare, but benign, disease has been associated

recently with a mutation of the human *cMOAT* gene [12]. More recent studies performed in heterozygous TR[−] rats and in *cMOAT*-transfected MDCKII cells have shown that the level of GSH excretion correlates with the expression level of the transporter, suggesting that cMOAT might serve an important physiological function in maintenance of bile flow as well as in hepatic GSH turnover [13].

The 2,4,5-trichlorophenoxyacetic acid herbicide (2,4,5-T), a chemical with anionic structure, has been used extensively in agriculture, but is currently banned from use owing to its possible carcinogenic and teratogenic activity [14]. This herbicide, as well as other chemicals like fibrates, plasticizers and compounds of natural origin such as fatty acids, is well known as a peroxisome-proliferator agent, particularly in the rodent liver [15]. We have reported previously that oral administration of these peroxisome-proliferator agents induces *mdr2* gene expression and its encoded P-glycoprotein isoform in the mouse liver and increases biliary phospholipid output [16,17]. Interestingly, we observed that 2,4,5-T also induced in mice a significant increase in bile flow [17]. This particular effect seems to be related to the bile-acid-independent flow fraction, since neither bile-salt output nor bile-salt-pool composition were modified in the experimental mice [17]. The significant increase in bile flow observed in 2,4,5-T-fed mice may therefore be caused by an increase in organic anion secretion or by an increase of GSH secretion, the major driving force of bile-acid-independent flow [18].

In the present study we hypothesized that the increase in bile

Abbreviations used: cMoat/cMOAT, mouse and human canalicular multispecific organic anion transporters, respectively; *GCS-HS*, γ -glutamylcysteine synthetase heavy-subunit gene; GY/TR[−] or TR[−] rat, Groningen yellow/transport-deficient rat; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); ACO, fatty acyl-CoA oxidase; CAT, carnitine acetyl-CoA transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LAP, leucine aminopeptidase.

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flow observed in 2,4,5-T-fed mice is related to the induction of *cMoat* expression and its encoded protein in the canalicular domain of the hepatocytes, and increased biliary GSH output. We also present evidence that the effect of 2,4,5-T on *cMoat* gene expression in the mouse liver is independent of the peroxisome-proliferation phenomenon.

EXPERIMENTAL

Animals and diet

Male CF I mice, weighing 25–28 g, were used in these studies. All mice received humane care, the guidelines for which are outlined in [19]. The animals were housed in wire-floored cages, with a 12-h light/dark cycle at 25 °C and given free access to a diet containing 20 % casein, 20 % sucrose, 5 % cellulose, 45 % starch, 5 % corn oil, 1 % vitamin mix, 0.3 % D,L-methionine and 3.5 % salt mix, prepared according to the recommendations of the American Institute of Nutrition. Experimental groups received for 4 days a similar diet supplemented with either 0.125 % 2,4,5-T (Sigma, St. Louis, MO, U.S.A., approx. 98 % w/w, without detectable levels of dioxin) or 0.3 % clofibrate (ICI Pharmaceuticals, Macclesfield, Cheshire, U.K.). Clofibrate and 2,4,5-T were dissolved in ethanol and acetone respectively, mixed with the diet, and the solvent evaporated at room temperature under a hood.

Human hepatoma cell culture

Human hepatoma cell line Huh7, derived from a hepatocellular carcinoma [20], and human hepatoma cell line HepG2, derived from a hepatoblastoma [21], were maintained at 37 °C under a 5 % CO₂ atmosphere in Dulbecco's modified Eagle's medium, containing 10 % fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin (all from Gibco, Grand Island, NY, U.S.A.). The cells were seeded at 25 % confluence in an 80-cm² flask in serum-free Dulbecco's modified Eagle's medium. The culture media were made up to have a final concentration of either 250 µM clofibrate or 125 µM 2,4,5-T, dissolved in DMSO and sonicated for 10 min. The final concentration of DMSO in the culture media was 0.1 %. The cell lines were incubated for 72 h and the medium was changed every 24 h.

Bile collection in 2,4,5-T- and clofibrate-treated mice

Bile flow and bile composition were analysed in 2,4,5-T- and clofibrate-treated animals as described previously [16,17]. On the day of the experiment, mice were anaesthetized with intraperitoneal pentobarbital (45 mg/kg; Nembutal, Abbott Laboratories, North Chicago, IL, U.S.A.). The common bile duct was cannulated with a PE10 polyethylene catheter (Clay-Adams, New York, NY, U.S.A.) and bile was collected for 30 min into preweighed Eppendorf tubes containing 100 µl of 5 % 5-sulphosalicylic acid to prevent oxidation of GSH to GSSG [22]. The mouse's body temperature was kept between 37 °C and 37.5 °C. Biliary bile acids were quantified as previously described [16,17]. After bile collection, liver samples were removed, weighed, rapidly frozen in liquid nitrogen and stored at –70 °C until processing for RNA extraction.

GSH and GSSG assay

The concentrations of GSH, GSSG and total glutathione (GSH plus GSSG) were measured in liver tissue and bile by the enzymic recycling procedure using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and glutathione reductase (DTNB-GSSG reductase

recycling assay) [23]. Liver tissue was removed rapidly, rinsed in 0.15 M NaCl, blotted dry and homogenized in 10 vols/g of tissue of 5 % sulphosalicylic acid. The homogenate was centrifuged at 1300 g for 10 min at 4 °C for protein precipitation. Liver supernatant and collected bile were centrifuged (12000 g for 3 min) and stored at 4 °C until they were assayed. To assay for total glutathione, 700 µl of 0.3 mM NADPH in stock buffer (143 mM sodium phosphate/6.3 mM Na-EDTA, adjusted to pH 7.5), 100 µl of 6 mM DTNB, 25 µl of sample and water to give a final volume of 1.0 ml were mixed in a cuvette and equilibrated to 30 °C. To the warmed solution, 10 µl of glutathione reductase (50 units/ml) was added and 5-thio-2-nitrobenzoic acid formation was monitored continuously at 412 nm until 2.0 absorbance units were reached. The GSH content of the aliquot assayed was determined by comparison of the rate observed with a standard curve generated using GSH. The oxidized fraction of total glutathione was measured after trapping the reduced fraction with vinylpyridine. Briefly, the 5-sulphosalicylic acid supernatant solution (100 µl) was placed in a 1.5-ml plastic tube and 2 µl of 2-vinylpyridine was added with mixing. The supernatant was then neutralized to pH 6.0–7.0 by the addition of triethanolamine. After 60 min, the derivatized samples were subjected to the DTNB-GSSG reductase recycling assay [23].

Measurement of enzymic activities

To assess the biological effect of peroxisome-proliferator agents on β -oxidation enzymes, the activities of the peroxisomal enzymes fatty acyl-CoA oxidase (ACO) and carnitine acetyl-CoA transferase (CAT) were measured in homogenates of human hepatoma cell lines, treated with 2,4,5-T or clofibrate [24].

Probes

Mouse *cMoat* partial cDNA probe was amplified from mouse liver RNA by reverse transcriptase PCR, based on the published sequence of rat *cMoat* [3]. The following primers, corresponding to conserved sequences, were used: primer upstream, 5'-GAC-TCTGACAACTTGAATGGGACC-3' (3098–3121); primer downstream, 5'-CCTTCTCTCCGCTCTTGATGTTAC-3' (4047–4070). The resulting PCR product, a 973-bp fragment, was sequenced by primer extension (Biot Core, Palo Alto, CA, U.S.A.), and showed over 85 % nucleotide identity to the rat *cMoat* cDNA. A 1163-nucleotide fragment of human *cMOAT* probe was also synthesized based on the communicated cDNA sequence (GenBank accession number U49248), using the following primers: upstream, 5'-AGCCTGAAGGAAGACGAA-GAAGTAG-3' (2913–2937); downstream, 5'-CCGATGTACC-AAGTGATCCCTC-3' (4054–4075). A 995-nucleotide fragment of the mouse γ -glutamylcysteine synthetase heavy subunit (GCS-HS) probe was also synthesized based on the communicated cDNA sequence (GenBank accession number AA409909). The cDNA probes were labelled to high specific radioactivity (1×10^9 d.p.m./µg of DNA) with [α -³²P]dCTP (3000 Ci/mmol; NEN Research Products, Boston, MA, U.S.A.) [25]. For the run-on assays, an ACO partial cDNA probe was also prepared by reverse transcriptase PCR, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1200-bp *EcoRI* fragment of the mouse cDNA.

RNA extraction

Total cellular RNA was extracted from mouse liver and Huh7 culture cells using the guanidinium-phenol method. The quantity and purity of the RNA were estimated spectrophotometrically

and the integrity was checked on a 1% agarose gel containing 2.2 M formaldehyde. Only samples in which the ribosomal RNAs appeared to be intact were used in the experiments. Poly(A)⁺-enriched RNA was isolated from total RNA using the Poly(A)⁺ Tract System IV from Promega (Madison, WI, U.S.A.).

Northern-blot analysis

Poly(A)⁺ RNA from mouse liver and 10 µg of total RNA extracted from exponentially growing human hepatoma cells were separated by electrophoresis on horizontal 1% agarose gels containing 2.2 M formaldehyde, and transferred on to nylon filters (NEN Research Products). The filters were hybridized as described previously [16,17]. Analysis of the autoradiograph was performed using Kodak X-Omat AR film at -70 °C. Densitometric analysis of the autoradiograph was performed using a CS-9000 scanning densitometer (Shimadzu, Kyoto, Japan) and the relative contents of the specific mRNA were expressed in arbitrary densitometric units.

Western-blot analysis

Liver plasma-membrane fractions enriched in the bile canaliculus domain were prepared from control and clofibrate- and 2,4,5-T-treated animals as described previously [17]. To assess similar enrichment in isolation of liver canaliculus membrane proteins, the relative amount of leucine aminopeptidase (LAP), a specific canaliculus membrane protein, was estimated by Western blotting using a polyclonal antibody.

Canaliculus membrane proteins (25 µg) were suspended in 30 µl of electrophoresis buffer containing 0.5% SDS and 10% glycerol, and electrophoresis in 8% polyacrylamide gel was carried out without heating the samples. For immunoblotting, the nitrocellulose membranes were incubated for 2 h in PBS/M/T buffer (PBS containing 5% skimmed milk and 0.05% Tween-20) and then with the cMOAT monoclonal antibody (mAb M2 III-5 hybridoma culture medium diluted 8-fold with PBS/M/T) [3]. Immunoreactivity was visualized with peroxidase-conjugated rabbit anti-mouse IgG by the enhanced chemiluminescence procedure (Amersham, Little Chalfont, Bucks, U.K.). LAP immunodetection was used as a loading control of liver canaliculus membrane proteins.

The human hepatoma Huh7 cultured cells were homogenized in sample buffer and 10 µg of proteins were separated by SDS/PAGE and immunoblotted as the mouse liver canaliculus membrane proteins.

Transcription rate *in vitro*: nuclear run-on assay

Hepatocytes were isolated by a modification of the two-step *in situ* collagenase perfusion method [26], and the nucleus-isolation procedure was performed as described previously [27]. The nuclei were resuspended in 600 µl of nuclear freezing buffer [50 mM Tris/HCl (pH 8.3)/40% glycerol/5 mM MgCl₂/0.1 mM EDTA]. An aliquot of nuclei was counted in a haemocytometer and the remaining resuspended nuclei were stored at -70 °C.

The transcription reaction was performed as described previously [27]. In parallel experiments, α-amanitin to a final concentration of 10 µg/ml was used in the transcription reaction to inhibit RNA polymerase II. Nuclear RNA was isolated using the guanidinium-phenol method. The probes of interest were denatured for 10 min at room temperature in a solution of 0.25 M NaOH/0.5 M NaCl, diluted in 0.1 × SSC/0.125 M NaOH (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and then applied to a nylon membrane using a Minifold system (Gibco BRL, Gaithersburg, MD, U.S.A.). The following probes

were used: *cMoat*, ACO and GAPDH, and the linearized ØX174 plasmid was used as a control for non-specific binding.

The membranes containing the bound probes were pre-hybridized at 65 °C for 2 h in a solution containing 0.1 M phosphate buffer (pH 7.0), 1% SDS, 10 mM EDTA, 0.3 M NaCl and 5 × Denhardt's solution. The hybridization was performed at 65 °C for 36 h in the same solution, which also contained 2.0 × 10⁷ c.p.m./ml of labelled RNA transcript. The membranes were then washed in three changes of 0.1 × SSC and 0.1% SDS at the hybridization temperature. Following autoradiography, the blots were quantified using a scanning densitometer and the relative contents of transcripts were expressed in arbitrary densitometric units.

Statistical analysis

All results are expressed as means ± S.D. A non-paired Student's *t* test program was used for comparison of differences between means. Values were considered to be significantly different if the *P* value was equal to or less than 0.05.

RESULTS

Effect of 2,4,5-T on bile secretion

Bile flow significantly increased in 2,4,5-T-treated mice, without changes in the biliary output of bile salts (Table 1). Remarkably, a significant increase in biliary GSH output was observed in 2,4,5-T-treated mice. In contrast, clofibrate did not induce changes in bile flow, bile salts or total glutathione output. In both the control and the 2,4,5-T-treated mice, around 70% of total glutathione excreted into the bile was in its reduced form (Table 1).

Hepatocellular concentrations of total glutathione were increased in 2,4,5-T-treated mice compared with control animals, indicating increased synthesis of GSH in experimental animals: total glutathione, 10.95 ± 0.84 versus 5.12 ± 0.47 mM; GSSG, 0.25 ± 0.01 versus 0.12 ± 0.02 mM (*n* = 3; *P* < 0.05).

Effect of 2,4,5-T and clofibrate on *cMoat* gene expression in mouse liver

The herbicide 2,4,5-T has some structural features in common with clofibrate, one of the most active peroxisome proliferators known [28]. The effect of both agents on *cMoat* gene expression was studied by Northern blotting. Analysis of *cMoat* mRNA levels in liver of 2,4,5-T- or clofibrate-treated mice showed a specific hybridization signal of about 8.0 kb (Figure 1). The size of the mouse *cMoat* mRNA detected was similar to the longest transcript previously found in rat liver [3]. A faint 6.5-kb band was also detected in some samples (Figure 1B), corresponding to the second transcript of *cMoat* gene reported previously in rat liver [3]. To confirm comparable RNA loading in each lane, the filter was also hybridized to the GAPDH cDNA probe (Figure 1). Increased levels of *cMoat* mRNA, expressed in arbitrary densitometric units, were observed in the liver of mice treated with the phenoxyacetic acid herbicide 2,4,5-T; 3.6 ± 0.05 versus control, 1.0 ± 0.4 (*n* = 3; *P* < 0.05). In contrast, clofibrate did not induce the *cMoat* mRNA levels; 1.6 ± 0.6 (*n* = 3; *P* > 0.05).

Expression of cMoat protein in the mouse liver

To determine whether increased hepatic *cMoat* mRNA was associated with increased expression of its encoded product, a Western blot was performed using proteins isolated from liver plasma-membrane fractions enriched in the bile canaliculus domain of 2,4,5-T-treated mice. A molecular-mass range between

Table 1 Effect of 2,4,5-T on bile composition

For 4 days, CFI mice were fed on a control diet supplemented with 0.125% 2,4,5-T or 0.3% clofibrate. Bile was collected for 30 min. The values are means $\pm$ S.D, $n = 5$. Units for bile-salt and all glutathione measurements were nmol/min per g of liver and, for bile flow, units were μ l/min per g of liver. Significant differences (where $P < 0.05$) compared with control values are marked with an asterisk. ND, not determined.

Treatment	Bile flow	Bile salts	Total glutathione	GSSG	GSH
Control	1.13 $\pm$ 0.15	34.05 $\pm$ 25.42	4.33 $\pm$ 1.00	1.68 $\pm$ 0.72	2.65 $\pm$ 0.34
Clofibrate	1.00 $\pm$ 0.15	33.78 $\pm$ 19.60	4.39 $\pm$ 0.85	ND	ND
2,4,5-T	2.23 $\pm$ 0.39*	39.70 $\pm$ 16.80	10.60 $\pm$ 3.82*	3.20 $\pm$ 1.05	7.40 $\pm$ 3.30*

Figure 1 Effect of 2,4,5-T and clofibrate on *cMoat* gene expression in the mouse liver

Groups of animals (three mice in each group) were fed for 4 days with 0.125% 2,4,5-T (A) or 0.3% clofibrate (B). Poly(A)⁺ RNA (5 μ g) isolated from liver of control and treated animals was hybridized by Northern blotting to a radiolabelled *cMoat* cDNA probe as described in the Experimental section. Control loading of poly(A)⁺ RNA is shown by the GAPDH blot. Molecular-size standards are indicated on the left in kb.

190 and 200 kDa was estimated for the immunoreactive signal of cMoat protein (Figure 2). The 68-kDa band seen in the Western blot is probably due to degradation of the 190–200-kDa band, as shown previously in a Western blot using canalicular membrane of rat liver [4]. Compared with control animals, the cMoat protein content increased 2.5 ($\pm$ 0.3)-fold in the hepatocytes of 2,4,5-T-treated mice. There were no apparent differences in protein loading on Western blots, assessed by the immunodetection of LAP, a specific marker of canalicular membrane proteins (Figure 2, lower panel). Clofibrate did not induce cMoat protein in the mouse liver (results not shown).

Figure 2 Effect of 2,4,5-T on cMoat protein expression in the mouse liver

Proteins (25 μ g) isolated from liver plasma-membrane fractions enriched in bile canalicular domain from control and 0.125% 2,4,5-T-treated mice were separated by SDS/PAGE and transferred on to a nitrocellulose filter. cMoat protein was immunodetected using a monoclonal antibody (mAb M2 III-5). LAP immunodetection was used as a loading control for canalicular plasma membrane proteins. Molecular-mass standards are indicated on the left in kDa.

Effect of 2,4,5-T on *cMoat* gene transcription rate

Further experiments were performed to explore the mechanism(s) responsible for 2,4,5-T-mediated induction of the *cMoat* gene. A nuclear run-on assay using nuclei isolated from the liver of control and 2,4,5-T-treated mice was used to examine relative transcription rates of the *cMoat* gene. As seen in Figure 3, there was a clear enhancement in the *cMoat* nuclear run-on hybridization signal. Densitometric analysis of the assays showed a 3.9 ($\pm$ 0.6)-fold increase for the *cMoat* hybridization signal, 3.3 ($\pm$ 0.2)-fold for ACO, used as a positive control for peroxisome proliferation, and no change for GAPDH, a gene of constitutive expression. Hybridization of labelled RNA transcripts to the ØX174 plasmid, used as a control of non-specific binding, was

Figure 3 Effect of 2,4,5-T on *cMoat* gene transcription rate

Nuclei were isolated from hepatocytes from control and 0.125% 2,4,5-T-treated mice and used for a nuclear run-on assay. The labelled transcripts were hybridized to partial cDNA probes. The probes used were *cMoat*, GAPDH, ACO and ϕ X174 plasmid vector. In parallel experiments, nuclei were pre-treated with 10 μ g/ml α -amanitin before transcription reaction. The nuclear run-on blots were quantified using a scanning densitometer and the relative contents of specific transcripts were expressed in arbitrary densitometric units. Three independent experiments of the nuclear run-on were performed.

Figure 4 Effect of 2,4,5-T on *GCS-HS* gene expression in the mouse liver

The Northern blot showing the effect of 2,4,5-T on *cMoat* gene expression in mouse liver (see Figure 1A) was stripped and re-hybridized to a radiolabelled *GCS-HS* cDNA probe as described in the Experimental section. Control loading of poly(A)⁺ RNA is shown by the GAPDH blot. Molecular-size standards are indicated on the left in kb.

not detected. Treatment of the nuclei with α -amanitin, which inhibits RNA II polymerase activity, markedly inhibited labelling of the *cMoat*, ACO and GAPDH genes, in both control and 2,4,5-T-treated mice.

Effect of 2,4,5-T and clofibrate on *GCS-HS* gene expression in the mouse liver

The reaction catalysed by GCS-HS is the rate-limiting step in GSH synthesis [29]. Thus further experiments were performed to assess the effect of 2,4,5-T or clofibrate on *GCS-HS* gene expression in the mouse liver. A Northern blot showed a specific hybridization signal of 3.2 kb (Figure 4). Increased levels of GCS-HS mRNA, expressed in arbitrary densitometric units, were observed in the liver of 2,4,5-T-treated mice; 2.5 ± 0.4 versus 1.0 ± 0.1 ($n = 3$; $P < 0.05$). Clofibrate, which did not modify biliary glutathione output, did not induce the *GCS-HS* gene either (results not shown).

Figure 5 Effect of 2,4,5-T on *cMOAT* gene and protein expression in Huh7 human hepatoma cell line

(A) Northern-blot analysis. Huh7 human hepatoma cells were seeded at 25% confluence and incubated for 72 h with 125 μ M 2,4,5-T or 250 μ M clofibrate. Total RNA (10 μ g) was hybridized by Northern blot to a radiolabelled human *cMOAT* cDNA probe. The equivalent loading of total RNA is shown by the GAPDH blot. (B) Western-blot analysis. Proteins (10 μ g) from Huh7 human hepatoma cells were electrophoresed through an SDS/PAGE gel and transferred on to a nitrocellulose filter. Human *cMOAT* protein was immunodetected using a monoclonal antibody (mAb M2 III-5). LAP immunodetection was used as a loading control. Molecular-mass and -size standards are indicated on the left.

Effect of 2,4,5-T and clofibrate on *cMOAT* gene expression in human hepatoma cell lines

Chemically induced peroxisome proliferation is not observed in human cells [28]. Therefore, to assess whether the 2,4,5-T-mediated induction of the *cMoat* gene in mice was independent of peroxisome proliferation, further experiments in human hepatoma cell lines were conducted. A Northern blot analysis of *cMOAT* mRNA in the Huh7 human hepatoma cell line showed a specific hybridization signal of 6.0 kb, corresponding to the human transcript (Figure 5A). To confirm comparable RNA loading in each lane, the filter was also hybridized to a GAPDH cDNA probe (Figure 5A). Increased levels of human *cMOAT* mRNA (4-fold, mean of two experiments) were observed in 2,4,5-T-treated Huh7 cells. In contrast, clofibrate did not induce the human *cMOAT* gene in this hepatoma cell line. Neither 2,4,5-T nor clofibrate induced the *cMOAT* gene in the HepG2 human hepatoma cell line (results not shown).

The human *cMOAT* protein content also increased 3.2-fold in the 2,4,5-T-treated Huh7 hepatoma cell line (mean of two experiments) compared with the control (Figure 5B). As expected, neither 2,4,5-T nor clofibrate induced changes in the enzymic activities of the peroxisomal enzymes ACO or CAT in the two human hepatoma cell lines used in these experiments (results not shown).

DISCUSSION

In this study we report for the first time that the *cMoat* gene is inducible in mice *in vivo* at the transcriptional level with significant functional consequences on biliary GSH secretion and bile flow. In several animal species, including humans, the bile-flow formation has two fractions: first, the fraction mostly related to bile-acid output is characterized by a linear relationship between bile-acid-secretion rates and bile flow; secondly, a bile-acid-independent fraction of bile flow is determined by the output of organic solutes into the bile, particularly GSH [18]. In the present study, the increased bile flow observed in 2,4,5-T-treated mice was most probably due to an increased output of GSH, the major driving force of bile-acid-independent flow [18]. Two lines of evidence support this interpretation: changes in bile-salt output in treated mice were not observed (Table 1) and the herbicide 2,4,5-T, an organic anion, is almost completely eliminated unchanged by urinary excretion in several species, including rodents [30]. Our observations of *cMoat* gene induction in mouse liver and increased biliary output of GSH agree with studies performed both *in vivo* and *in vitro*, which demonstrate the role of *cMoat* in the excretion of GSH [13,31]. In rats homozygous for the *cMoat* mutation (TR^- rats), bile-acid-independent flow is significantly decreased and GSH secretion is absent from bile [31]. More recently, Paulusma et al. [13] have shown that, in rats heterozygous for the *cMoat* mutation, biliary GSH excretion was decreased by 63% of control rats, and human *cMOAT*-transfected MDCKII cells displayed a more than 10-fold increase in apical GSH excretion compared with wild-type MDCKII cells [13]. In that study, they concluded that *cMOAT* mediates low-affinity transport of GSH and, since hepatocellular GSH concentrations are high, the *cMOAT* transporter might serve an important physiological role in maintenance of bile flow as well as in hepatic GSH turnover [13]. Interestingly, it has been also shown in yeast that the orthologue of mammalian MRP1 and *cMOAT*/MRP2 proteins, YCF1, mediates ATP-dependent low-affinity transport of GSH, supporting the hypothesis that GSH efflux from mammalian cells is mediated by these membrane proteins [32]. However, we cannot exclude in our study the possibility that additional canalicular transport proteins, not yet

identified, may also be responsible for the increased output of GSH observed in the bile of 2,4,5-T-treated animals [33].

Although the herbicide 2,4,5-T can induce several peroxisomal-protein-encoding genes [15], the biological effect on the *cMoat* gene seems to be independent of peroxisome proliferation. In fact, clofibrate, one of the most active peroxisome proliferators, did not induce the *cMoat* gene in mouse liver (Figure 1B). On the other hand, the herbicide 2,4,5-T also induced the human *cMOAT* gene and its encoded protein in the Huh7 cell line (Figure 5) without changes in the enzymic activities of ACO or CAT, used as markers of peroxisome proliferation [24]. Although xenobiotic-mediated peroxisome proliferation is a conserved phenomenon in taxonomic groups as evolutionarily distant as plants and mice, it has not been observed in primates, including humans [28]. In this context, our experiments suggest that the peroxisome-proliferation phenomenon is probably not required for 2,4,5-T-mediated induction of *cMoat* and that both events are unrelated.

The increased *cMoat* mRNA level found in our study was explored at a molecular level by a nuclear run-on assay using isolated nuclei (Figure 3). Assuming that nuclear run-on assays *in vitro* correlate with the rate of gene transcription *in vivo*, our results suggest that the overexpression of the *cMoat* gene by this xenobiotic is regulated mainly at a transcriptional level. Further studies are needed to define the transcription factors and the regulatory regions of the *cMoat* gene involved in 2,4,5-T-mediated induction.

There is accumulating evidence in the literature that regulation of the *GCS-HS* gene is a major determinant of cellular levels of GSH, the most abundant antioxidant in cells [29]. The increased levels of hepatocellular GSH observed in 2,4,5-T-treated animals prompted us to study the expression of this gene in our experimental model. As shown in Figure 4, the increased synthesis of hepatocellular GSH found in 2,4,5-T-treated mice was due to induction of the *GCS-HS* gene. Interestingly, this finding represents the first evidence of *GCS-HS* gene modulation mediated by this xenobiotic. Many conditions are known to influence the steady-state mRNA levels of the *GCS-HS* gene. These conditions fall into several categories, which include drug-resistant tumour cell lines, growth-related factors such as hormones, antioxidants, the possibility of cell GSH itself and oxidative stress [29]. Thus we can speculate that the increased synthesis of hepatocellular GSH found in 2,4,5-T-treated animals, as a result of the up-regulation of the *GCS-HS* gene, may represent an additional protective mechanism to control the consequences of oxidative stress induced by this xenobiotic in the liver.

In addition, our findings represent the first evidence of co-induction of *GCS-HS* and *cMoat* genes in hepatocytes mediated by a xenobiotic. In this respect, it is of interest to note that the MRP1 conjugate transporter, the homologue of the *cMOAT* protein in other tissues, and the *GCS-HS* gene are frequently co-expressed in varying conditions such as in drug-resistant tumour cells [34], untreated tumour cells and normal mouse tissues [35]. Although the mechanisms by which *GCS-HS* and *MRP1* are coordinately expressed in various cell sources are not clear, it has been hypothesized that, particularly in situations where cytotoxic xenobiotics are used, oxidative-stress-induction mechanisms may play a role in the regulation of these genes through the activation of oxidative-stress-responsive transcription factors, such as nuclear factor κB or the activator protein 1 family [35]. In fact, the promoter regions of both *GCS-HS* and *MRP1* genes contain a putative activator protein 1-binding site [36]. Recently it has also been shown that 5'-regulatory regions of the *cMoat* gene contain binding sites for this transcription-factor family [37]. Further experiments are needed to examine the transcription factors and the *cis*-acting regulatory elements in the promoter regions

responsible for the co-ordinate regulation of *cMoat* and *GCS-HS* genes in hepatocytes.

Recent evidence has shown that xenobiotics like the chemical carcinogen 2-acetylaminofluorene and the anti-neoplastic drug cisplatin induce the *cMoat* gene in primary cultures of rat hepatocytes [38]. According to these observations, it has been proposed that the overexpression of cMoat transporter may be part of an adaptive response preventing further cell damage [38]. It is still unclear, however, which consequences of 'cell stress' and/or DNA damage are responsible for the *cMoat* gene induction observed in rat hepatocytes. The biological effects of these agents on biliary GSH secretion *in vivo* and on *GCS-HS* gene modulation remain to be defined, to better understand the co-ordinate regulation of both genes.

In summary, this study has revealed for the first time a model in which the multispecific organic anion transporter gene, *cMoat*, is co-induced with the *GCS-HS* gene *in vivo* in mouse liver, associated with increased expression of cMoat protein, increased GSH synthesis and biliary GSH output. These observations may represent co-ordinate changes in the expression of protective genes in the liver in response to xenobiotic exposure.

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