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Impact of dimethyl sulfoxide on expression of nuclear receptors and drug-inducible cytochromes P450 in primary rat hepatocytes☆

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Abstract

Dimethyl sulfoxide (DMSO) is reported to induce hepatocyte redifferentiation. The impact of DMSO on liver transcription factors, cytochromes P450 (CYPs), and nuclear receptors regulating CYP expression was assayed in primary rat hepatocytes by QPCR. CYP 2B1, 3A1, and 4A1 mRNAs were reduced to 10–30% of initial liver levels without DMSO and restored at or above liver levels by DMSO treatment. In contrast, CYP1A1 mRNA increased ~5-fold during the course of culture, independent of DMSO. DMSO enhanced expression of the nuclear receptors CAR, PXR, and PPARα 2- to 5-fold, which may contribute to the increase in basal CYP expression. Without DMSO, liver transcription factors were decreased (HNF4, C/EBPα), largely unchanged (HNF1α, HNF3α, and C/EBPβ) or elevated (HNF3β, HNF6) compared to intact liver. DMSO largely restored hepatic levels of HNF4 and C/EBPα, partially suppressed the elevated levels of HNF6, increased HNF1α ~2-fold, and had little effect on HNF3α, HNF3β, and C/EBPβ. Overall, DMSO helped maintain normal hepatic transcription factor patterns and basal CYP and nuclear receptor profiles, suggesting that hepatocytes cultured with DMSO may be useful for CYP metabolic studies under conditions where the endogenous liver phenotype is preserved.

Keywords: Dimethyl sulfoxide; Nuclear receptor; Cytochrome P450; Primary rat hepatocyte; Liver-enriched transcription factor

Cytochrome P450 (CYP)[1] enzymes belonging to gene families 1–4 carry out oxidative metabolism of structurally diverse xenobiotics and lipophilic endobiotics, many of which induce CYP gene expression. Hepatic expression of CYP1A1 can be dramatically increased by exposure to β-naphthoflavone (BNF), which acts through the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (Arnt) to induce CYP1A1 gene transcription [1]. Other drug-inducible CYPs are constitutively expressed in the liver at low levels and are dramatically induced following exposure to prototypic drugs, such as phenobarbital (CYP2B), dexamethasone (CYP3A), and ciprofibrate (CYP4A). Induction of these CYPs is mediated by specific receptors belonging to the nuclear receptor superfamily: constitutive androstane receptor (CAR), in the case of CYP2B [2], pregnane X receptor (PXR) for CYP3A [3], and peroxisome proliferator-activated receptor-α (PPARα) for CYP4A [4]. These nuclear receptors are activated upon binding their foreign chemical ligands, which leads to heterodimerization with the retinoid X receptor (RXR) and binding to cognate DNA response elements upstream of target genes, followed by activation of gene transcription [5].

Primary hepatocyte cultures serve as a very useful in vitro model for studies of hepatic drug metabolism and xenobiotic activation [6–8]. However, one of the pitfalls of this system is that primary hepatocytes readily dedifferentiate and thereby lose liver-specific functions during the course of culture. Previous studies have shown that when primary rat hepatocytes are cultured with dimethyl sulfoxide (DMSO), certain liver-specific

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[1] *Abbreviations used:* AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CAR, constitutive androstane receptor; C/EBP, CCAAT/enhancer binding protein; CYP or P450, cytochrome P450; DMSO, dimethyl sulfoxide; HNF, hepatocyte nuclear factor; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RXR, retinoid X receptor.

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functions are preserved [9,10]. Other studies demonstrate significant improvements in responsiveness to phenobarbital and other classic CYP inducers in rat hepatocytes cultured in modified Chee's medium on a Vitrogen substrate bound covalently to the culture dish [11]. These inductions are achieved without the need for Matrigel overlay [11,12]. Modified Chee's medium is also superior in terms of hepatocyte viability and function [13], overall yield of microsomal protein, and the ultra-structural features of hepatocyte monolayers [14,15]. In the present study, we investigate whether hepatocytes cultured in modified Chee's medium in the presence of DMSO present an advantage with respect to constitutive expression of the nuclear receptor-targeted CYPs 1A1, 2B1, 3A1, and 4A1 or the responsiveness of these CYPs toward prototypic foreign chemical inducers. We also investigate the impact of DMSO on the expression of liver-enriched transcription factors and nuclear receptors that regulate CYP gene expression.

Materials and methods

Materials

Type II collagenase (263 activity units/mg) (Worthington Biochemical, Lakewood, NJ), Vitrogen (Cohesion, Palo Alto, CA), β-naphthoflavone, phenobarbital, dexamethasone, ciprofibrate, thymidine, epidermal growth factor, hepatocyte growth factor, transferrin, insulin, selenium (Sigma Chemical, St. Louis, MO), L-glutamine and modified Chee's medium (Formulation No. 88-5046EA, Gibco-BRL, Grand Island, NY), and TRIzol reagent (Invitrogen, Carlsbad, CA) were obtained from the sources indicated.

Rat primary hepatocyte isolation and primary cell culture

Adult male Fischer 344 rats, 150–220 g (Taconic, Germantown, NY), were anesthetized with ketamine and xylazine. Primary hepatocytes were isolated using a two-step collagenase perfusion method [16]. Livers were perfused at 29 ml/min, first with Ca^{2+}-free perfusion buffer (142 mM NaCl, 6.7 mM KCl, and 10 mM Hepes, pH 7.4) for 4 min, and then with perfusion buffer containing 0.54 mg/100 ml type II collagenase and 70 mg/100 ml $CaCl_2 \cdot 2H_2O$ for 3–4 min. Livers were then dissected out and dispersed to single cells in ice-cold, modified Chee's medium on ice. Cell viability was evaluated by trypan blue exclusion. Hepatocyte preparations with viability $\geqslant 90\%$ were plated in 6-well plates (Falcon 353046, BD Labware, Franklin Lakes, NJ) precoated with Vitrogen using a carbodiimide coupling procedure [11] at 7×10^5 cells/well. Culture medium was changed 4 h after the cells were plated to remove any unattached cells. Cells were cultured in modified Chee's medium containing 0.1 μM dexamethasone, 3.7 g/L sodium bicarbonate, 10 mg/L thymidine, 4 mM L-glutamine, transferrin (6.25 μg/ml), insulin (6.25 mg/L), and selenium (6.25 ng/ml) [11], with the addition of 10 ng/ml epidermal growth factor, and 1 ng/ml hepatocyte growth factor. DMSO was added to the culture medium at a final concentration of 2% (v/v) beginning on day 4 and maintained for the duration of each experiment, typically 9–12 days. Medium was changed twice a week for all the experiments.

CYP induction studies

Hepatocytes were treated with 20 μM β-naphthoflavone, 1 mM phenobarbital, 10 μM dexamethasone or 100 μM ciprofibrate for a 72-h period beginning on culture day 6, i.e., 2 days after addition of 2% DMSO to the cells. Control cultures received vehicle only. Triplicate culture wells were assayed at each time point and the resultant data are presented as mean ± SD values. Each experiment was repeated at least twice to verify the reproducibility of the results with different batches of hepatocytes.

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions for monolayer cells. Total RNA was prepared from single wells of a 6-well culture dish with a typical yield of 10–20 μg per well. Reverse transcription to yield cDNA was carried out using GeneAmp RNA PCR core kit (Applied Biosystems, Foster City, CA) using 1 μg of total hepatocyte RNA in a total volume of 20 μl according to the manufacturer's instructions. DNA primers (Table 1) were designed using Primer Express software (Applied Biosystems). Quantitative real-time PCR (QPCR) mixtures contained 8 μl SYBR Green PCR master mix (Applied Biosystems), 0.3 μM of each PCR primer, and 4 μl of 1:20 to 1:100 diluted cDNA in a total volume of 16 μl. Three aliquots of 5 μl each of the 16 μl master mix were loaded into each of three separate wells of a 384-well plate to evaluate the reproducibility of the QPCR. Samples were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Results were analyzed using the comparative C_T ($\Delta\Delta C_T$) method, as described in User Bulletin 2 of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, P/N 4303859 Rev. A). The amount of each target mRNA, normalized to an 18S RNA endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta C_T}$. The ΔC_T value of the liver cDNA pool was used as a calibrator to calculate the fold-difference between each sample and the liver cDNA pool unless indicated otherwise. The liver cDNA value was set as 1

Table 1
Real-time PCR primers

Oligo No.	Gene	Accession No.	Sequence (5′–3′)	Amplicon size (bp)	Orientation
ON-1047	CYP1A1	NM–012540	cct gga gac ctt ccg aca ttc	70	Forward
ON-1048	CYP1A1		ggg ata tag aag cca ttc aga ctt g		Reverse
ON-973	CYP2B1	J00719	gct caa gta ccc cca tgt cg	109	Forward
ON-974	CYP2B1		atc agt gta tgg cat ttt act gcg g		Reverse
ON-977	CYP3A1	M10161	agg cac ctc cca cct atg ata c	144	Forward
ON-978	CYP3A1		tgg gca taa aca cac cat tga		Reverse
ON-979	CYP4A1	M14927	ggt tct ttg ggc aca agc a	104	Forward
ON-980	CYP4A1		gct tcc cca gaa cca tcg a		Reverse
ON-981	AHR	NM–013149	ggg cca aga gct tct ttg atg	102	Forward
ON-982	AHR		gca agt cct gcc agt ctc tga		Reverse
ON-983	ARNT	NM–012780	tgg gct caa gaa gat cgt tca	107	Forward
ON-984	ARNT		tcc att cct gca tct gtt cct		Reverse
ON-985	CAR	NM–022941	cca cgg gct atc att tcc at	101	Forward
ON-986	CAR		ccc agc aaa cgg aca gat g		Reverse
ON-987	PPARα	NM–013196	tct ccc cac ttg aag cag atg	102	Forward
ON-988	PPARα		tct cct ctc cga ggg act ga		Reverse
ON-991	PXR	AF–151377	gac ggc agc atc tgg aac tac	112	Forward
ON-992	PXR		tga tga cgc cct tga aca tg		Reverse
ON-1051	RXRα	NM–012508	gtg cct gga gca cct gtt ct	75	Forward
ON-1052	RXRα		ctc cag cat ctc cat gag gaa		Reverse
ON-1053	RXRβ	M81766	gcc caa atg acc cag tga ct	108	Forward
ON-1054	RXRβ		tcg tcc aga ggt agg gag gaa		Reverse
ON-842	18S	X01117	cgc cgc tag agg tga aat tc	138	Forward
ON-843	18S		cca gtc ggc atc gtt tat gg		Reverse
ON-904	HNF1α	X54423	aca cct ggt acg tcc gca ag	51	Forward
ON-905	HNF1α		cgt ggg tga att gct gag c		Reverse
ON-902	HNF3α	X55955	aac ccc agt gcc gaa tca c	51	Forward
ON-903	HNF3α		gct agc ctt tcc gtg cac ac		Reverse
ON-900	HNF3β	L09647	gac cct gca ccc tga ctc tg	51	Forward
ON-901	HNF3β		cgc agg tag caa ccg ttc tc		Reverse
ON-894	HNF4	X57133	tgg caa aca cta cgg agc ct	51	Forward
ON-895	HNF4		ctg aag aat ccc ttg cag cc		Reverse
ON-896	HNF6	X96553	aag ccc tgg agc aaa ctc aa	51	Forward
ON-897	HNF6		cca cat cct ccg gaa agt ctc		Reverse
ON-908	C/EBPα	X12752	gcg caa gag ccg aga taa ag	51	Forward
ON-909	C/EBPα		ttc tgc tgc gtc tcc acg t		Reverse
ON-898	C/EBPβ	NM–24125	cgc ctt tag acc cat gga ag	51	Forward
ON-899	C/EBPβ		agg cag tcg ggc tcg tag tag		Reverse

and the abundance of each hepatocyte cDNA sample was then calculated in terms of the fold-difference, except as noted. Student's *t* test was applied to samples for statistical analysis, with p values <0.05 considered significant (GraphPad Prism software, v 4.0).

Validation of QPCR data

QPCR analyses were performed to measure the efficiencies of the target and the reference amplifications and to validate the $\Delta\Delta C_T$ method. The efficiencies of all target amplifications using the primers designed in this study were approximately the same as that of the endogenous reference, 18S ribosomal RNA, with the absolute value of the slope of log input amount vs ΔC_T being less than 0.1 (data not shown). In cases where the value was greater than 0.1, a new set of primers was designed until the value met this requirement. The specificities of the primers were examined using the disassociation curve analysis function included in the ABI PRISM 7700 QPCR software package and by agarose gel electrophoresis to verify the size of each amplicon (data not shown).

Results

Optimization of concentration and time of DMSO addition

In a series of initial studies, DMSO was added to the hepatocyte culture medium at a concentration of 1 or 2% beginning on day 0, 2 or 4 of cell culture. DMSO at 2% (v/v) beginning on day 4 was the most effective at restoring normal liver levels of several liver-enriched transcription factors, such as HNF4 (data not shown),

in agreement with another study [10]. This protocol was therefore used for the entire study. To optimize the matrix for hepatocyte attachment, we compared Vitrogen and rat tail collagen I covalently bound to the plates in the presence of carbodiimide. Both matrices were equally effective with respect to expression of liver-like levels of HNF4 (data not shown). Vitrogen was chosen for use in all subsequent experiments.

Expression of CYP mRNAs

CYP1A1 mRNA was undetectable in cDNA prepared from a pool of eight individual untreated male rat livers. CYP1A1 mRNA was barely detectable in rat primary hepatocytes cultured on day 0, i.e., 4 h after cell plating, when the cells first attached to the plate. Beginning 24 h later (day 1 cells), CYP1A1 mRNA

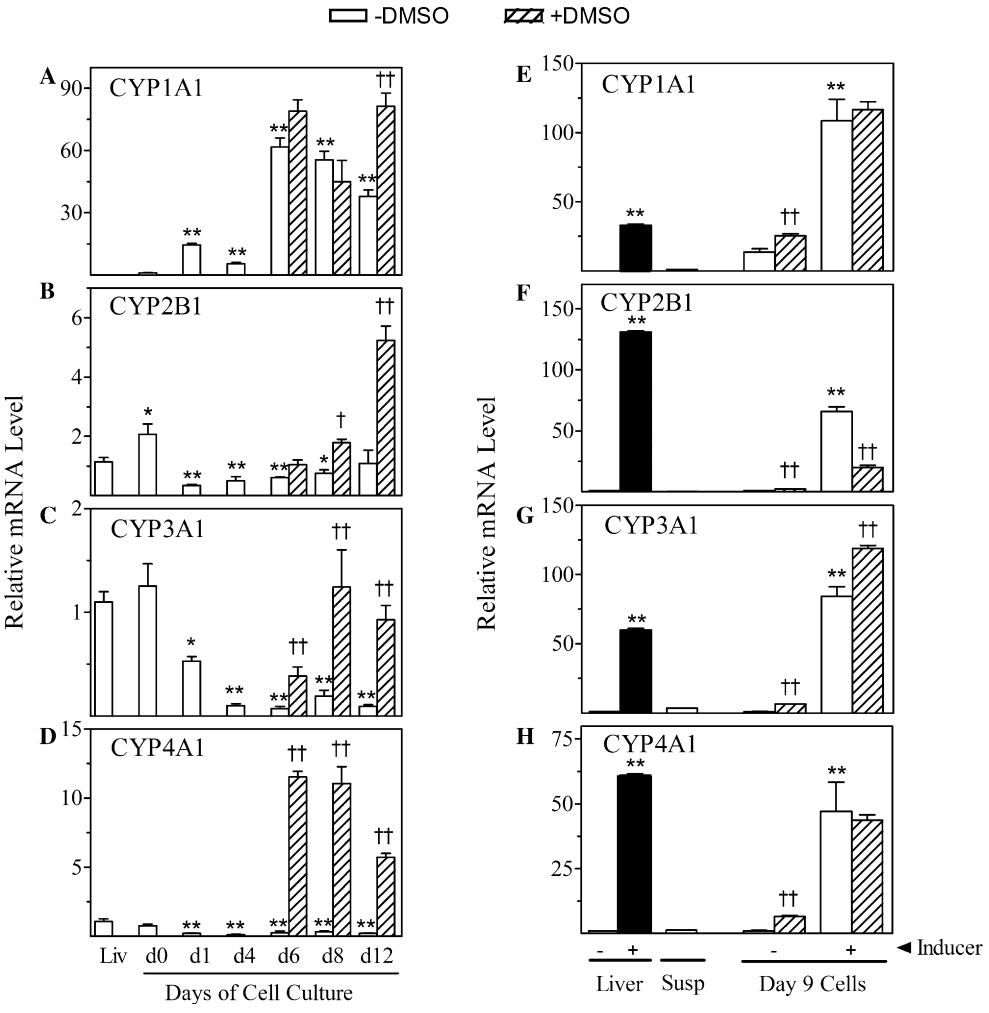

Fig. 1. Expression and induction of CYP mRNAs in primary rat hepatocyte cultures: impact of DMSO treatment. (A–D) QPCR analysis of hepatocyte RNA samples prepared from an uninduced male rat liver cDNA pool (Liv) or from hepatocytes cultured from day 0 (4 h after plating) to day 12, in the presence or absence of 2% DMSO added on day 4, as described under Materials and methods. Statistical analysis: cultured hepatocytes without DMSO vs intact liver (*$p < 0.05$; **$p < 0.01$); hepatocytes cultured with DMSO vs hepatocytes cultured without DMSO ($^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$). (E–H) The first two bars represent relative mRNA levels in untreated and P450 inducer-induced rat liver, respectively (filled bars). Adult male rats were administered the inducers β-naphthoflavone (E; 40 mg/kg/day for 4 days, i.p.), phenobarbital (F; 80 mg/kg/day for 4 days, i.p.), dexamethasone (G; 100 mg/kg/day for 4 days, i.p.) or ciprofibrate (H; 20 mg/kg/day for 7 days, i.p.), respectively. Susp, relative RNA levels in the original suspension of primary hepatocytes on day 0, prior to plating; Day 9 cells, cells were untreated or were given P450 inducer treatment from day 6 to day 9, as indicated, and then harvested on day 9. Cells used in this experiment were cultured without (clear bars) or with 2% DMSO added on day 4 (shaded bars). The P450 inducers applied to the hepatocyte cultures were β-naphthoflavone (20 μM), phenobarbital (1 mM), dexamethasone (10 μM), and ciprofibrate (0.1 mM), for (E)–(H), respectively. Statistical analysis: inducer-treated rat liver vs untreated rat liver (*$p < 0.05$; **$p < 0.01$); inducer-treated hepatocytes vs untreated hepatocytes (*$p < 0.05$; **$p < 0.01$); and hepatocytes cultured with DMSO vs hepatocytes cultured without DMSO ($^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$). For (A)–(H), ΔC_T values determined for the uninduced liver cDNA pool were used as reference values and were set = 1 for all genes analyzed, except for CYP1A1, where the liver cDNA was not detectable and the ΔC_T value of day 0 hepatocytes was used as reference in calculating relative mRNA levels. Data shown for the cultured hepatocytes are means ± SD values for $n = 3$ individual hepatocyte culture wells prepared from a single rat liver, whereas data for the intact livers represent means ± SD values for $n = 3$ triplicate QPCR analyses carried out on a pool of adult male rat livers ($n = 8$ uninduced livers; $n = 2$–3 induced livers per treatment group).

gradually increased and reached its maximum level on day 6 of culture (Fig. 1A). Addition of DMSO on day 4 did not have a major effect on the level of CYP1A1 mRNA, except that there was a 2-fold increase in CYP1A1 expression day 12 in the DMSO-treated cultures. In contrast, CYP 2B1, 3A1, and 4A1 mRNAs were each detectable in the uninduced liver cDNA pool and in day 0 hepatocytes. However, each of these CYP mRNAs decreased significantly within the first day after cell plating and was maintained at ~10–30% of the initial liver levels from day 4 to day 12 (Figs. 1B–D). Addition of DMSO to the cultures on day 4 markedly increased expression of all three CYPs, restoring basal expression (CYP3A1) or increasing it up to 5- to 10-fold

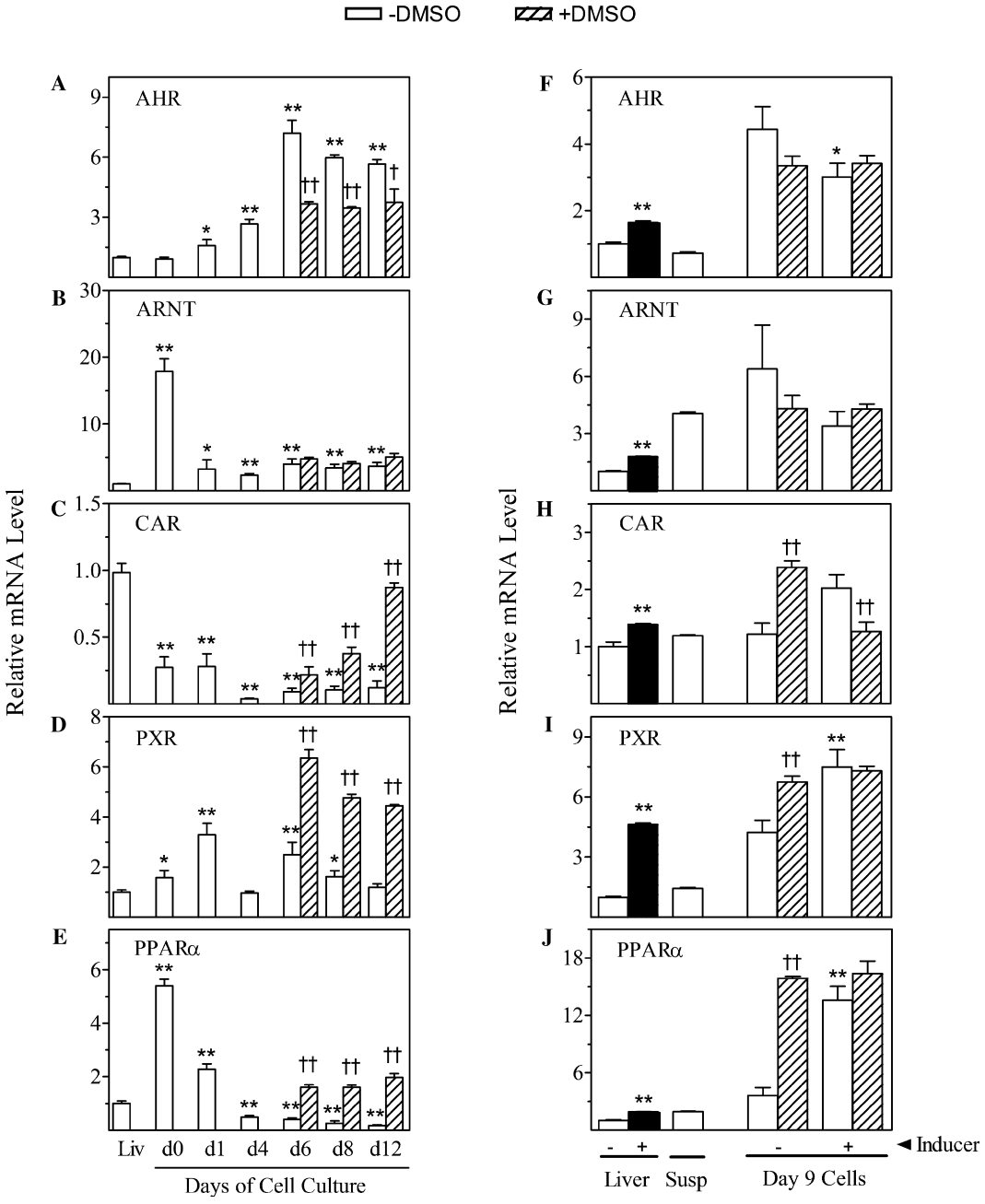

Fig. 2. Effect of DMSO on expression and induction of nuclear receptor mRNAs in primary rat hepatocyte cultures. QPCR analysis of hepatocyte RNA samples prepared from intact liver cDNA (Liv or Liver) or from primary hepatocytes cultured from day 0 to day 12, in the presence or absence of 2% DMSO (A–E), or cultured for 9 days, with or without P450 inducing agents added from days 6 to 9, as detailed in Fig. 1. Analyses shown in (A)–(E) were carried out using the same samples shown in Figs. 1A–D. Analyses shown in (F)–(J) used the same samples shown in Figs. 1E–H. ΔC_T values for the uninduced liver cDNA pool were used as reference values to calculate relative RNA levels, as described in Fig. 1. See Fig. 1 legend for other details.

higher (CYPs 2B1, 4A1) from day 6 to 12, i.e., beginning 2 days after DMSO addition (Figs. 1B–D). The high levels of expression of all four CYP genes were maintained in the DMSO-treated cells through the course of the experiment (12 days).

Treatment of rat hepatocytes with the classic CYP inducers, β-naphthoflavone (20 μM), phenobarbital (1 mM), dexamethasone (10 μM), and ciprofibrate (100 μM), led to dramatic increases in the expression of CYPs 1A1, 2B1, 3A1, and 4A1, respectively (Figs. 1E–H). The induction levels achieved in the cultured cells were generally comparable to the induced levels of each CYP achieved in livers of rats treated with the same inducer in vivo (cf. first two sets of bars, panels E–H). Although DMSO raised the basal levels of CYP 2B1, 3A1, and 4A1 mRNAs, it had little effect on the maximum inducibility of these CYPs. Thus, the fold-induction of each CYP mRNA relative to uninduced liver was largely the same in cells cultured in the absence of DMSO as in the presence of DMSO (Figs. 1E–H; last pair of bars of each panel). One notable exception was CYP2B1, which was more highly induced by phenobarbital in the absence of DMSO (Fig. 1F).

Impact of DMSO on expression of nuclear receptors

We next examined the expression of the four major xenoreceptors that regulate hepatic CYP mRNA induction by foreign chemicals. AhR mRNA gradually increased during the course of culture and reached its highest level on day 6 (Fig. 2A). This pattern was similar to that of CYP1A1 mRNA (Fig. 1A), whose levels are regulated by AhR in combination with Arnt. DMSO reduced the level of AhR mRNA by ~30–40% (Fig. 2A). The level of Arnt mRNA, on the other hand, decreased over the first 24 h in culture, to about 10–30% of the day 0 level, and subsequently was maintained at this level regardless of the DMSO status (Fig. 2B). Notably, the levels of Arnt mRNA in hepatocytes were 2- to 3-fold higher than in normal intact liver at all time points, except day 0 cells, where the level was inexplicably 15- to 20-fold higher. DMSO enhanced expression of the nuclear receptors CAR, PXR, and PPARα 2- to 5-fold, which parallels the increased basal expression of their respective target genes, CYPs 2B1, 3A1, and 4A1 (Figs. 2C–E vs Figs. 1B–D). By contrast, the mRNA levels of RXRα and RXRβ, which heterodimerize with CAR, PXR, and PPARα, were only modestly affected (<2-fold changed) by the presence of DMSO (Figs. 3I and J). Treatment of the cells with CYP inducers had minimal effect on nuclear receptor mRNA levels ($\leqslant$2-fold changes), independent of the presence of DMSO (Figs. 2F–I), with the exception of PPARα, which increased ~5-fold upon ciprofibrate treatment in the absence of DMSO (Fig. 2J).

Expression of liver-enriched transcription factors in DMSO-induced hepatocytes

Liver-enriched transcription factors, such as HNF1α and HNF4, play a key role in determining the liver specificity of CYP gene expression [17]. We therefore investigated the impact of DMSO on the expression of seven major HNF RNAs. With the exception of an increase in expression seen in day 1 cells, HNF1α mRNA did not change dramatically over a 12-day period in cells cultured in the absence of DMSO. Addition of DMSO resulted in a ~2-fold increase in HNF1α mRNA compared to the level of intact liver or day 0 cells, as seen in the day 6, 8, and 12 cultures (Fig. 3A). HNF4 mRNA decreased by 2- to 3-fold during the course of culture compared to intact liver levels and was restored back to near-normal liver levels by DMSO treatment (Fig. 3B). HNF3α and HNF3β mRNAs were substantially decreased in day 0 cells compared to intact liver and were subsequently increased back to ~70% (HNF3α) or ~200% of intact liver levels (HNF3β) (Figs. 3C and D). HNF6 mRNA was also increased substantially, to levels up to 9-fold higher than intact liver, whereas this increase was substantially moderated in the presence of DMSO (Fig. 3G). Basal levels of C/EBPα mRNA decreased by 70% during the 12-day cell culture period, whereas C/EBPβ mRNA levels remained unchanged. DMSO restored C/EBPα mRNA to ~60–75% of the levels found in intact liver and day 0 hepatocytes, but had no effect on C/EBPβ mRNA (Figs. 3E and F).

Discussion

The rapid loss of liver-specific enzyme activities and metabolic functions has been a major factor limiting the utility of primary hepatocytes as an in vitro model for liver function. To circumvent this problem, efforts have been made in several laboratories to establish a culture system that maintains hepatocytes in a differentiated state [18]. Our previous studies, culturing rat hepatocytes in modified Chee's medium on covalently bound Vitrogen-coated plates, demonstrated the advantages of this system, both for long-term cell maintenance and for achieving strong, reproducible induction of CYP2B1 in response to phenobarbital treatment [11,19]. These methods have been adopted by others for studying hepatic CYPs and their regulation in rodent hepatocytes [12,15,20,21] and also human hepatocytes [22]. However, the profiles of expression of HNFs and nuclear receptors, both of which are essential for liver CYP gene expression, were not previously investigated. The present studies demonstrate that inclusion of 2% DMSO in modified Chee's culture medium beginning on day 4 not only induces the re-differentiation seen in another culture medium [23], but also restores near-normal liver

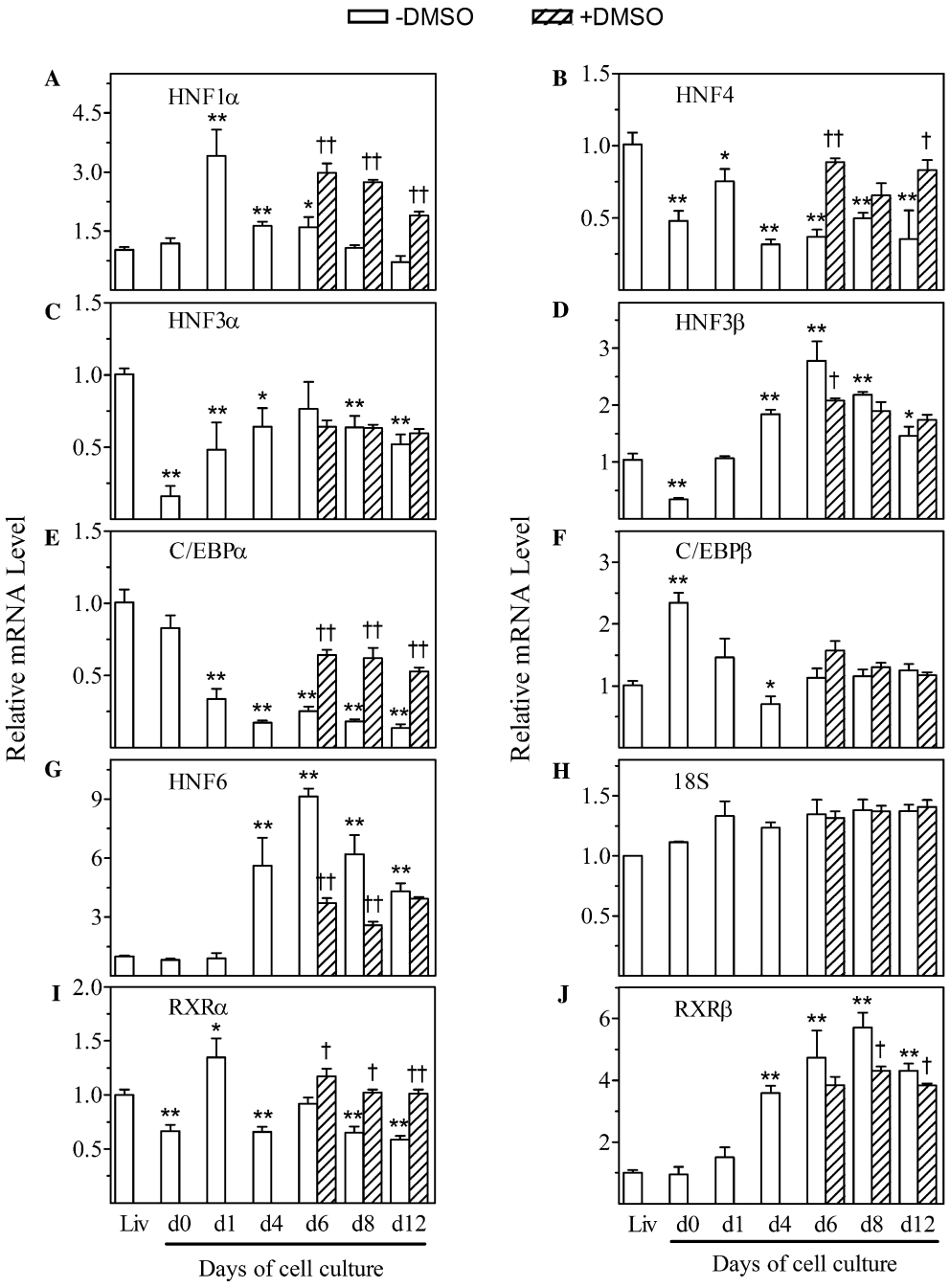

Fig. 3. Expression of liver-enriched transcription factor and RXRα and RXRβ mRNAs in primary rat hepatocyte cultures. QPCR analysis of hepatocyte RNA samples prepared from intact liver cDNA (Liv) or from primary hepatocytes cultured from day 0 to day 12, in the presence or absence of 2% DMSO (A–E), as detailed in Fig. 1. Analyses were carried out on the same samples shown in Figs. 1A–D. ΔC_T values for the uninduced liver cDNA pool were used as reference values to calculate relative RNA levels, as described in Fig. 1. See Fig. 1 legend for other details.

levels of several HNFs and nuclear receptors important for liver CYP expression.

DMSO has been used as a differentiation-inducing agent for many tumor cell lines [24]. However, the mechanism by which DMSO induces the differentiation of tumor cells and certain other cell types is poorly understood. In the case of HL60 cells, DMSO-induced differentiation is associated with downregulation of telomerase [25] and apurinic/apyrimidinic endonuclease activities [26], and transient formation of DNA strand breaks [27]. DMSO enhances albumin and α-fetoprotein production in transformed hepatocytes and hepatocarcinoma cells [28,29] and helps maintain normal adult rat hepatocytes in the differentiated state, as indicated by the production of liver-specific plasma proteins, including the consistent production and secretion of albumin at high levels [9]. Furthermore, the morphology of DMSO-treated hepatocytes resembles cells isolated

from normal liver. DMSO-treated hepatocytes have also been characterized with respect to other liver-specific functions [30–32]. In a further development, Mitaka and co-workers established that re-differentiation and restoration of several liver functions could be induced by DMSO in primary rat hepatocytes plated at sub-confluent levels when cultured with L15 medium supplemented with 10 ng/ml epidermal growth factor [10,23]. In the present study, we used QPCR technology in combination with gene-specific primers to characterize the effects of DMSO added to hepatocytes cultured in modified Chee's medium, and found that DMSO supports the expression of several HNFs and nuclear receptors important for regulating the expression of drug-metabolizing P450s.

Liver-enriched transcription factors, such as HNF1α, HNF4, C/EBPα, and C/EBPβ, play important roles in regulation of hepatic CYP expression [17]. HNF1α acts as a positive regulator for expression of CYP genes 1A2, 2E1, and 7A1 [33–35]. CYP4A1 mRNA is significantly increased in HNF1α null mice, perhaps due to enhanced lipolysis and increased production of fatty acid activators of PPARα, which in turn induce CYP4A1 expression [36]. In the present study, however, DMSO treatment dramatically increased PPARα and CYP4A1 mRNA levels in association with an increase, and not a decrease, in HNF1α mRNA. HNF4 and C/EBPα are important positive regulators of *CYP3A* and *CYP2B* genes, respectively [17], and correspondingly, our results showed increased expression of CYP3A1 and CYP2B1 mRNA in DMSO-treated hepatocytes in association with increased expression of HNF4 and C/EBPβ. The nuclear receptors PXR and CAR, which, respectively, regulate expression of these CYPs, were also increased. Using modified Chee's medium in the absence of DMSO, HNF levels did not decrease as dramatically as reported by Mizuguchi et al. in L15 medium (decreases of ~30% (HNF1α) and ~60% (HNF4) relative to intact liver levels (this study) vs $\geqslant 90\%$ decreases reported previously [10]). Nevertheless, DMSO substantially increased expression of these and other HNFs, restoring the overall profile close to that of intact liver.

The DMSO-induced restoration of liver-like profiles of liver-enriched transcription factors and nuclear receptors correlated with DMSO-enhanced expression of several specific liver CYP genes. Inclusion of DMSO did not affect the maximal level of CYP expression achieved in cells treated with CYP-inducing drugs and chemicals, however, suggesting that the xenoreceptors required for CYP induction are not limiting for induction when cells are stimulated with high concentrations of receptor ligands. Thus, the basal level of expression of nuclear receptors is sufficient to support large increases in CYP expression. In contrast, nuclear receptor levels appeared to be an important determinant of the level of CYP expression in the absence of added CYP inducers: basal levels of the inducible CYPs 2B1, 3A1, and 4A1 were increased in association with significant DMSO-induced increases in the receptors CAR, PXR, and PPARα, respectively. This increase in basal CYP expression may result from an increased sensitivity to low concentrations of endogenous cellular CYP inducers due to the increase in 'spare receptors.' In the case of AhR and CYP1A1, substantial increases in expression compared to liver were also observed in the absence of added inducer, beginning on day 6 of culture; however, the increases were independent of the presence of DMSO.

A large fraction of drugs in clinical use today are metabolized by enzymes belonging to CYP gene families 2, 3, and 4 [37]. These CYPs are liver-enriched or liver-specific in their expression and many of them are inducible at the level of transcription. CYP induction can be of clinical significance in terms of its impact on drug–drug interactions and pharmacokinetics [38]. Primary hepatocytes have the potential to serve as useful models to study the effects of CYP induction, and for extrapolation of in vitro data on CYP-dependent drug metabolism to liver cells in vivo [39,40]. The present finding that DMSO treatment restores basal expression of CYP2B1 and CYP3A1 to close to normal liver levels suggests that this culture system may provide a useful cellular model for studying CYP2B- and CYP3A-dependent drug and other foreign chemical metabolism under conditions where the endogenous liver phenotype is preserved. Although improved in this regard, the culture model described here does not provide for fully normal liver expression of all rat CYPs, as indicated by the high level of CYP4A1 expression and by the fact that DMSO treatment did not lead to restoration of several rat CYP2C mRNAs (unpublished data). Further modifications of the culture conditions, including evaluation of the effects of lower DMSO concentrations, may be useful in this regard (cf. impact of 0.5% DMSO on CYP expression in primary human hepatocytes [41]). Nevertheless, this culture model may be suitable for screening libraries of chemicals to better define pharmacophores that induce CYP2B or CYP3A expression and thus have the potential to contribute to drug–drug interactions.

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