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Repression of Cytochrome P450 Activity in Human Hepatocytes in Vitro by a Novel Hepatotrophic Factor, Augmenter of Liver Regeneration[S]

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ABSTRACT

Pathological disorders of the liver were shown to be associated with an impairment of hepatic drug metabolism mediated in part by growth factors. Augmenter of liver regeneration (ALR) is a novel liver-specific hepatotrophic growth factor, whereas its action on cytochrome P450 (P450) metabolism is completely unknown. Application of ALR to primary human hepatocytes in vitro reduced P450 isoenzyme activities (1A2 and 2A6) in a dose-dependent manner. Time-course analysis revealed that the maximal inhibitory effect was reached after 24 to 72 h of exposure with 50 nM ALR. The reduction of basal activities upon ALR treatment was 35% for CYP1A2, 56% for CYP2A6, 18% for CYP2B6, and 45% for CYP2E1. Additionally, after induction of P450 with specific inducers, ALR revealed an inhibitory effect on the isoenzyme activities (CYP1A2, 41%; CYP2B6, 35%). Investigations of protein and mRNA expression of basal and induced CYP1A2 and CYP3A4 after ALR treatment by Western blotting and real-time reverse transcriptase-polymerase chain reaction, respectively, suggest a regulation on the transcriptional level. Furthermore, ALR treatment increased nuclear factor kB activity and reduced constitutive androstane receptor but not pregnane X receptor or aryl hydrocarbon receptor expression. In contrast, ALR revealed no effects on phase II reactions (glutathione/oxidized glutathione, UDP-glucuronyltransferase conjugation). Our results indicate that ALR, as a member of hepatotrophic factors, down-regulates basal and induced P450 in human liver and therefore cross-links growth signals to regulation of hepatic metabolism. These findings further imply a possible role of ALR in drug interactions during impaired hepatic function, whereas liver regeneration is triggered.

Hepatic oxidative and reductive metabolism of endogenous and xenobiotic substrates is supported by a family of heme-containing monooxygenases known as cytochrome P450. The substrates metabolized by P450 include environmental pollutants, agrochemical, plant toxins, steroids, prostaglandins, and fatty acids. Furthermore, in humans, P450 play a central role in phase I drug metabolism and, therefore, are mainly responsible for drug interactions and individual drug metabolism (Danielson, 2002). In addition to detoxification, the versatile enzymes are also facilitating the production of reactive metabolites acting as highly reactive oxygen species, procarcinogens, or genotoxic xenobiotics (Murray, 2000; Villeneuve and Pichette, 2004). P450 expression and activation are mainly regulated by inducing agents, such as the substrates themselves or at a constitutive level (Danielson, 2002).

Homeostatic metabolic function is mostly retained after damage of the liver, but if injury is more severe, liver insufficiency might be accompanied by multiorgan failure (Barie, 1998). Hepatic dysfunction in vivo is difficult to quantify,

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ABBREVIATIONS: P450, cytochrome(s) P450; IL, interleukin; TNF, tumor necrosis factor; HGF, hepatocyte growth factor; EGF, epidermal growth factor; ALR, augmenter of liver regeneration; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kB; MC, 3-methylcholanthrene; Rif, rifampicin; rhALR, recombinant human ALR; EROD, 7-ethoxyresorufin *O*-deethylase; BROD, 7-benzoxyresorufin *O*-debenzylase; CH, coumarin 7-hydroxylase; PNP, *p*-nitrophenol hydroxylase; UGT, UDP-glucuronyltransferase; GSH, glutathione; GSSG, oxidized glutathione; PCR, polymerase chain reaction; AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; PXR, pregnane X receptor; RT, reverse transcriptase; ELISA, enzyme-linked immunosorbent assay.

often based on a multiplicity of causes, and may affect other organs depending on the liver-derived substrates (Barie, 1998). Pathological disorders of the liver including tissue injury, inflammation, cirrhosis, and tumor growth were shown to be associated with an impairment of hepatic drug metabolism, particularly the one mediated by P450 (Tygstrup et al., 2002; Shibuya et al., 2003; Villeneuve and Pichette, 2004). Factors of the so-called acute phase mediators, e.g., IL-6, IL-1, and TNF-α as well as growth factors like HGF and EGF, were identified to down-regulate P450 expression (Muntane-Relat et al., 1995; Greuet et al., 1997; Donato et al., 1998; De Smet et al., 2001; Tinel et al., 2003).

On the other hand, these cytokines (IL-6, IL-1, TNF-α) and growth factors (HGF, EGF) are highly expressed upon liver damage and were shown to play an important role within the process of liver regeneration (Michalopoulos and DeFrances, 1997). Recently, a novel human hepatotrophic growth factor, augmenter of liver regeneration (ALR; Hagiya et al., 1994; hematopoietin, He et al., 1993; Wang et al., 1999), derived from LaBrecque's hepatic stimulator substance (LaBrecque et al., 1987), has gained increased scientific interest (Gandhi et al., 1999; Wang et al., 1999; Tanigawa et al., 2000; Li et al., 2000, 2001; Chen et al., 2003). The 15-kDa polypeptide was reported to accumulate in animal studies after partial hepatectomy (Gandhi et al., 1999; Wang et al., 1999) and under circumstances of liver regeneration (Gandhi et al., 1999; Tanigawa et al., 2000). Furthermore, we could demonstrate increased ALR protein and mRNA expression in livers from patients with cirrhosis and hepatocellular and cholangiocellular carcinoma compared with normal livers (Thasler et al., 2005). Moreover, recombinant ALR was shown to stimulate proliferation of hepatocytes as well as hepatoma cells in vitro, and this hepatotrophic effect may be exerted through a specific receptor found on the plasma membrane (Wang et al., 1999; Li et al., 2000, 2001; Chen et al., 2003). Additionally, it was proposed that interaction of extracellular ALR with its receptor is responsible for enhanced proliferation and augmentation of liver regeneration by activating the mitogen-activated protein kinase (MAPK) cascade (MAPK pathway) (Li et al., 2000; Chen et al., 2003).

It is not clear yet whether ALR as a hepatotrophic factor and promising highly liver-specific agent additionally is involved in modulation of hepatic drug metabolism and whether ALR has a role in regulating P450 activity. Therefore, we investigated the effect of recombinant human ALR on primary human hepatocytes in vitro with regards to drug metabolism. Here, we demonstrate that ALR besides its function as an augmenter of hepatocyte proliferation also down-regulates basal and induced P450 activities in human liver presumably due to its ability to modulate transcription factors (NFκB) as well as nuclear receptors.

Materials and Methods

Reagents. Human recombinant HGF was purchased from R&D Systems (Minneapolis, MN). Collagenase (type IV), 7-ethoxyresorufin, 7-benzoxyresorufin, coumarin, 7-hydroxycoumarin, MC, Rif, FAD, HEPES, *p*-nitrophenol, resorufin, 4-methylumbilliferone, 3,3′-methylene-bis(4-hydroxycoumarin), and isopropyl-β-D-1-thiogalactopyranoside and other buffer supplements were purchased from Sigma Chemie (Deisenhofen, Germany). β-Glucuronidase/arylsulfatase was purchased from VWR (West Chester, PA). Collagen type I was purchased from Roche Diagnostics (Mannheim, Germany), and fetal calf serum was purchased from Biochrom (Berlin, Germany). Dulbecco's modified Eagle's medium with 4.5 g/l glucose was obtained from Cambrex Bio Science Verviers S.p.r.l. (Verviers, Belgium), and all other medium additives were purchased from Serva (Heidelberg, Germany).

Hepatocyte Preparation and Culture. Tissue samples from human liver resection were obtained from patients undergoing partial hepatectomy. Experimental procedures were performed according to the guidelines of the charitable state-controlled foundation (Human Tissue and Cell Research), with the informed patient's consent approved by the local ethical committee of the University of Regensburg. A total of 32 liver specimen from patients (12 females and 20 males; age ranged from 32 to 87, 60.88 $\pm$ 12.67 years) were used. Human hepatocytes were isolated using a modified two-step EGTA/collagenase perfusion procedure as described previously (Weiss et al., 2003). Viability of isolated hepatocytes was determined by trypan blue exclusion, and cells with a viability $>$ 85% were used for cell culture. Cells were plated on a collagen gel layer (1.0 mg/ml type I collagen) at a density of 1.0×10^5 cells/cm^2 in appropriate volume of culture media. The medium consisted of Dulbecco's modified Eagle's medium with 5% fetal calf serum, 2 mM L-glutamine, and supplements as follows: 1.7 mU/ml insulin, 3.75 ng/ml hydrocortisone, 100 μg/ml streptomycin, and 100 U/ml penicillin. After 16 h of plating, medium was replaced by medium without serum. Cells were incubated at 37°C in a humidified incubator with 5% CO_2, and media were changed daily unless otherwise stated. Viability of hepatocytes during culture period was monitored by cell morphology (light microscopy, image analysis) and determination of enzyme release into culture medium (aspartate aminotransferase activity).

Preparation of Recombinant Human ALR and Treatment of Cultures. Recombinant human ALR (rhALR) was prepared as described recently (Thasler et al., 2005). Fractions containing rhALR protein were combined and dialyzed against dialysis buffer (25 mM HEPES, 0.1% Tween 20, and 1 mM EDTA, pH 8.2) at 4°C, with a 3-fold buffer change. Afterward, rhALR protein was concentrated using a 5-kDa cut-off ultrafree-15 centrifugal filter device (Millipore Corporation, Billerica, MA).

HGF and ALR were prepared as sterile solutions in culture medium and were added to cultures at the indicated times. Treatments of cells started 24 h after plating followed changing culture medium. Controls were not treated and not induced cells. Specific P450 inducers MC and Rif were dissolved in dimethyl sulfoxide and added directly to the cells at the indicated time points to a final concentration of 2 and 50 μM (solvent concentration was lower than 0.5% v/v), respectively.

Measurement of Hepatic Enzyme Activities. Hepatic enzyme activities were determined as described elsewhere with slight modifications (Donato et al., 1998). For determining EROD and BROD activities, intact cultured hepatocytes were incubated with culture medium supplemented with 10 μM 3,3′-methylene-bis(4-hydroxycoumarin) and 8.3 μM 7-ethoxyresorufin or 15 μM 7-benzoxyresorufin for 60 min at 37°C, respectively. To 300 μl of medium supernatant, 100 μl of 100 U of β-glucuronidase/arylsulfatase in 0.1 M sodium acetate buffer, pH 4.5, was added and incubated for 2 h at 37°C, and the reaction was stopped by 1:3 dilution with ethanol. The resulting resorufin was quantified fluorometrically in microtiter plates (Genios Plus; Tecan Deutschland GmbH, Crailsheim, Germany) with 530 and 590 nm for excitation and emission, respectively. CH activity measurement was performed incubating cultured hepatocytes with 100 μM coumarin for 60 min at 37°C followed by treatment of 200 μl of medium supernatant with 50 μl of 100 U of β-glucuronidase/arylsulfatase in 0.1 M sodium acetate buffer, pH 4.5, for 2 h at 37°C. The reaction was stopped by 1:3 dilution with 0.1 M Tris, pH 9.0, and the resulting 7-hydroxycoumarin was quantified fluorometrically in microcuvettes with 355 and 460 nm for excitation and emission, respectively (SFM25; Bio-Tek Instruments, Winooski, VT). For determination of PNP activity, cultured hepatocytes were incubated with 0.5 mM *p*-nitrophenol for 60 min at 37°C. Reaction

was terminated adding 100 μl of trichloroacetic acid (55%) to the well, and cells and medium were scrapped into an Eppendorf tube followed by centrifugation at 10,000*g* for 10 min. Supernatant was diluted (1:11) with 10 M NaOH, and *p*-nitrocatechol was quantified immediately by measuring the absorption in a UV-visible spectrophotometer (Ultrospec 2000; GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 546 nm. To assess UGT activity, cultured hepatocytes were treated with 100 μM 4-methylumbelliferone, and at 15, 30, 45, and 60 min of incubation at 37°C, 10-μl aliquots of medium supernatant were transferred to a microtiter plate. After the addition of 190 μl of NaOH (10 mM) to each well, the remaining 4-methylumbelliferone was determined fluorometrically in a microplate reader (Genios Plus; Tecan Deutschland GmbH) with 355 and 460 for excitation and emission, respectively. The GSH/GSSG ratio was determined with a commercial available kit according to the manufacturer's instructions (Bioxytech GSH/GSSG-412; tebu-bio GmbH, Offenbach, Germany).

Analysis of P450 Protein Expression. Polyclonal goat anti-human CYP1A1/2 and rabbit anti-human CYP3A4 antisera were purchased from BD Biosciences (San Jose, CA). S-9 fractions from human cultured hepatocytes incubated with or without inducers as well as ALR for the indicated times were prepared (Holme et al., 1983) and dissolved in solubilization buffer (50 mM Tris/HCl, 0.5% SDS, and 1 mM EDTA), pH 6.8 [containing protease inhibitor: 1 μg/ml each chymostatin, leupeptin, antipain, and pepstatin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride]. Proteins were electrophoresed (30 μg of protein/lane) on a 10% SDS-polyacrylamide gel electrophorese gel followed by protein transfer to a 0.45-μm polyvinylidene difluoride membrane (PALL GmbH, Dreieich, Germany). Sheets were incubated with specific antisera against CYP1A2 or CYP3A4 (1:2000 with 4% dried milk) followed by incubation with horseradish peroxidase-labeled donkey anti-goat IgG (0.04 μg/ml in 4% dried milk) or goat anti-rabbit IgG (0.12 μg/ml with 4% dried milk) antibodies, respectively. Blots were developed using an enhanced chemiluminescence detection kit (Perbio Science GmbH, Bonn, Germany). For protein determination, the bicinchoninic acid assay (Pierce Chemical, Rockford, IL) was used with bovine serum albumin as reference.

Quantification of mRNA Expression by Real-Time RT-PCR. Total RNA was isolated from cultured hepatocytes using the RNeasy kit (QIAGEN GmbH, Hilden, Germany). First-strand cDNA was synthesized using 1 μg of total RNA and the avian myeloblastosis virus-reverse transcription reaction (Promega, Madison, WI). Transcript levels of human CYP3A4, CYP1A2, AhR, CAR, PXR, and β-actin were quantified using the real-time RT-PCR technology (LightCycler; Roche Diagnostics) with specific sets of primers (designed with the GeneTool Lite software; BioTools Inc., Edmonton, AB, Canada) based on published sequences: CYP3A4, forward, 5′-ATG AAC ATG AAA CTT GCT CTA AT-3′, and reverse, 5′-GAG CTC AAT GCA TGT ACA GAA TC; CYP1A2–3′, forward, 5′-CAA GGG ACA CAA CGC TGA-3′, and reverse, 5′-CCA GGA CTT CCC CGA TAC A-3′; AhR, forward, 5′-ACA TCA CCT ACG CCA GTC GC-3′, and reverse, 5′-TCT ATG CCG CTT GGA AGG AT-3′; CAR, forward, 5′-TGA TCA GCT GCA AGA GGA GA-3′, and reverse, 5′-AGG CCT AGC AAC TTC GCA TA; PXR, forward, 5′-CCA GGA CAT ACA CCC CTT TG-3′, and reverse, 5′-CTA CCT GTG ATG CCG AAC AA-3′; and β-actin, forward, 5′-CTA CGT CGC CCT GGA CTT CGA GC-3′, and reverse, 5′-GAT GGA GCC GCC GAT CCA CAC GG-3′. For PCR, 1 to 3 μl of cDNA preparation, 2.4 μl of 25 mM $MgCl_2$, 0.5 μM of forward and reverse primer, and 2 μl of SybrGreen LightCycler Mix (Roche Diagnostics) in a total of 20 μl were applied. The following PCR program was performed: 60 s at 95°C (initial denaturation); 20°C/s temperature transition rate up to 95°C for 15 s, 10 s at 58°C, 22 s at 72°C, and 10 s at 82°C acquisition mode single, repeated for 40 times (amplification). $MgCl_2$ concentration and annealing temperature were optimized for each primer set. The PCR reaction was evaluated by melting curve analysis following the manufacturer's instructions and checking the PCR products on 1.8% agarose gels. Each quantitative PCR was performed at least in duplicate for two sets of RNA preparations.

Quantification of Activated Nuclear NFκB Concentration. Nuclear extracts were prepared as previously described (Muhlbauer et al., 2004) with protease inhibitors in the form of the complete minitablets (Roche Diagnostics). Activated NFκB was quantified in nuclear extracts with the ELISA-based kit TransAm from Active Motif Inc. (Carlsbad, CA) according to the manufacturer's instructions, as described recently (Muhlbauer et al., 2004). ELISA-plates are coated with oligonucleotide (5′-GGGACTTTCC-3′) coding for an NFκB consensus site. Plates were preincubated with a binding buffer containing dithiothreitol and herring sperm DNA. Nuclear extracts (20 μg), solved in 20 μl of lysis buffer containing dithiothreitol and protease inhibitors, were added per well incubated at room temperature for 1 h. After a washing step, an anti-RelA (p65) antibody was added and incubated for another hour at room temperature. After an additional washing step, a horseradish peroxidase-conjugated secondary antibody was added followed by an additional 1 h of incubation at room temperature. After a last washing step, developing solution was added and the absorption was measured at 450 nm.

Statistical Analysis. Results represented in figures and tables are of representative experiments ($n \geq 3$ different donors) and were run in triplicate. Data are expressed as means ± S.D., and the statistic was performed by analysis of variance and Fisher's least significant difference test. Differences were considered significant when $p < 0.05$.

Results

ALR Reduces P450 Activity in Human Hepatocytes Dependent on Culture Period and Concentration. ALR, known to obtain augmenting potential on liver regeneration, was used to evaluate its impact on hepatic metabolism. Therefore, we determined P450 activities dependent on the timeframe of treatment during culture period and the concentration of ALR. Human hepatocytes were cultured either under control conditions or were incubated with 5 nM ALR for indicated times of the culture. After incubation with ALR, CYP1A2 activity, assessed as EROD activity, was measured and calculated as percentage with respect to EROD activity after 24 h of culture (Fig. 1). A time-dependent decrease of EROD activity during ALR treatment was observed with a

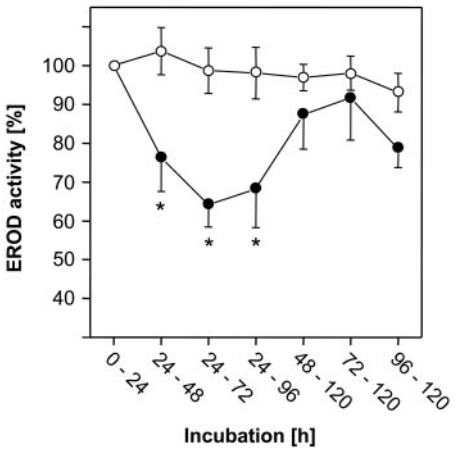

Fig. 1. Modulation of P450 activity by ALR dependent on culture period of primary human hepatocytes. Isolated hepatocytes were plated, maintained in culture, and treated without (control, open circle) or with (full circle) 5 nM ALR for the indicated times of the culture period. After the incubation period, EROD activity (CYP1A2) was measured and compared with activity of nontreated cells after 24 h in culture. *, $p < 0.05$, differs from control (three independent experiments in triplicate, ± S.D.).

maximum for a 24- to 72-h and 24- to 96-h incubation of culture period. This reduction in EROD activity was only detected if treatment started 24 h after plating, whereas any start time later gained a lower reduction of EROD activity compared with untreated cells (data not shown). Furthermore, the reduction of P450 activity after ALR treatment was dependent on the ALR concentration, which is demonstrated by a dose response-dependent reduction of CH (CYP2A6) and EROD (CYP1A2) activity (Fig. 2). Maximum reducing effect of ALR is reached at 50 nM, depressing CH and EROD activity to approximately 40 and 60% of nontreated cells, respectively.

ALR Reduces Basal and Induced (Phase I) P450 Activity. The effect of ALR on the basal activities of several P450 isoenzymes (CYP1A2, CYP2A6, CYP2B6, CYP2E1) of different donors were investigated. Hepatocytes were treated after a 24-h plating period either with standard medium or with 50 nM ALR for 72 h followed by P450 activity assays, which are based on specific substrate oxidations. Basal activities of a larger pool of different donors were investigated (Fig. 3, A and B) and showed a broad range of activities in the control group (CH, 89.95 ± 71.12 pmol/min 10^6 cells; EROD, 2.80 ± 1.77 pmol/min 10^6 cells) as well in the ALR group (CH, 46.20 ± 57.33 pmol/min 10^6 cells; EROD, 1.84 ± 1.16 pmol/min 10^6 cells). However, despite this interindividual variation, we observed a significant overall reduction of activities in the ALR group for CH and EROD for approximately 55.3 ± 21.2 and 34.5 ± 16.0%, respectively. Further ALR-treated hepatocytes (Fig. 4, A and B) demonstrated a reduction in BROD (CYP2B6) and PNP (CYP2E1) activity of approximately 18.1 ± 7.2 and 44.9 ± 26.4%, respectively.

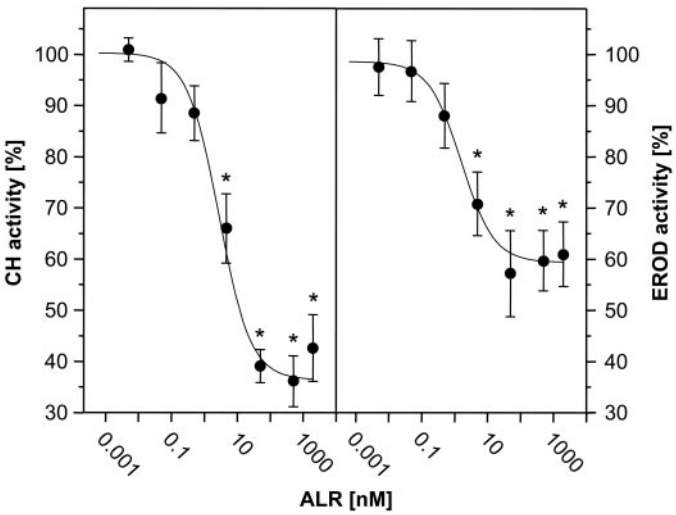

Fig. 2. Modulation of P450 activity dependent on ALR concentration. Twenty-four hours after plating, hepatocytes were treated for the consecutive 72 h with increasing concentrations of ALR (0.005–2000 nM) followed by CH activity (CYP2A6) and EROD activity (CYP1A2) determination. *, $p < 0.05$, differs from nontreated cells (100%). Four independent experiments in triplicate, ± S.D.

Furthermore, we analyzed the impact of ALR treatment on the inducibility of P450 isoenzymes. Again, hepatocytes were treated without or with 50 nM ALR for a 24- to 96-h culture period, and for the last 24 h of ALR treatment, specific inducers MC (2 μM) and PB (2 mM) were added, followed by P450 activity determination (Fig. 5, A and B). P450 isoenzymes of individual donors showed a high variety of inducibility but demonstrated also a significant overall reduction of 40.6 ± 10.1% and 34.7 ± 7.1% for induced EROD and BROD, respectively. Interestingly, the effect of ALR on CYP1A2 seemed to be independent of the rate of induction as seen by the low S.D. and almost similar reduction rate at basal CYP1A2 activity.

In addition to the effect of ALR on phase I, P450-dependent monooxygenase reactions, we also evaluated parameters of phase II reactions. We determined the UGT conjugation reaction and intracellular ratios of GSH/GSSG of hepatocytes incubated with 50 nM ALR for a 24- to 96-h culture period (Table 1). We observed some interindividual differences within the control and ALR group as well as variations of the effect of ALR on UGT activity and GSH/GSSG ratio without significance. Therefore, evidence of a modulating effect of ALR on hepatic metabolism is given for phase I (inhibition of P450) but not for the investigated phase II reactions.

ALR Reduces Basal and Induced P450 Protein and mRNA Expression. To investigate whether the observed reduction of basal and induced P450 activities by ALR is regulated via P450 protein expression, we performed Western blot analysis. Experiments were performed as described above for basal and induced P450 activities with MC and Rif as a specific inducer for CYP1A2 and CYP3A4, respectively. Cell lysates were immunoblotted with specific antisera, and results were quantified by densitometry (Fig. 6). ALR reduced basal and MC-induced CYP1A2 protein expression of approximately 30% (Fig. 6, A and B) as it was demonstrated

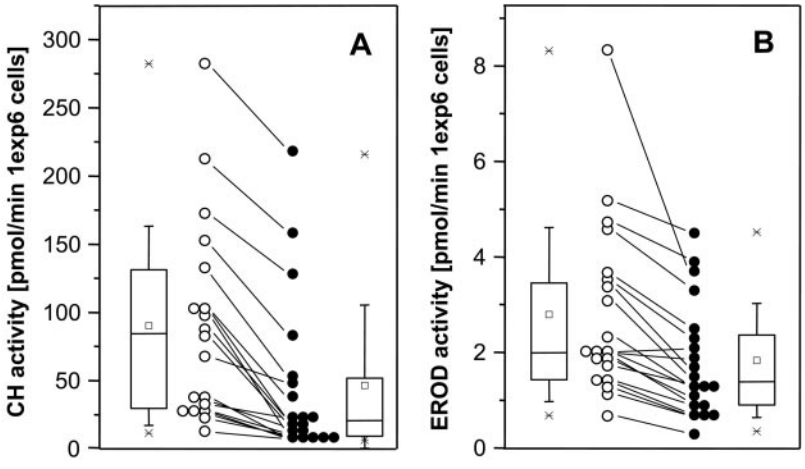

Fig. 3. Modulation of P450 activity of primary human hepatocytes by ALR. Twenty-four hours after plating, cultured hepatocytes from different donors were incubated without (control, open circle) or with (full circle) 50 nM ALR for 72 h followed by P450 activity measurement. The graphs show CH (A) and EROD (B) activities of ALR-treated and untreated cells of matched pairs of individual donors ($n = 19$) as well as a box plot with mean ± S.D., median, and 25/75 percentile. Experiments are shown in triplicate.

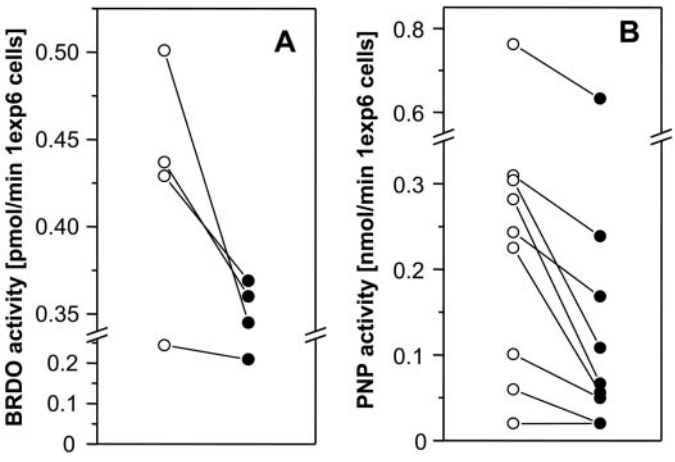

Fig. 4. Modulation of P450 activity of primary human hepatocytes by ALR. Cells from different donors cultured for 24 h were exposed either to standard medium (control, open circle) or medium with 50 nM ALR (full circle) for the consecutive 72 h. At 96 h of culture, BROD (CYP2B6, n = 4; A) and PNP (CYP2E1, n = 9; B) activities were determined. Experiments are shown in triplicate.

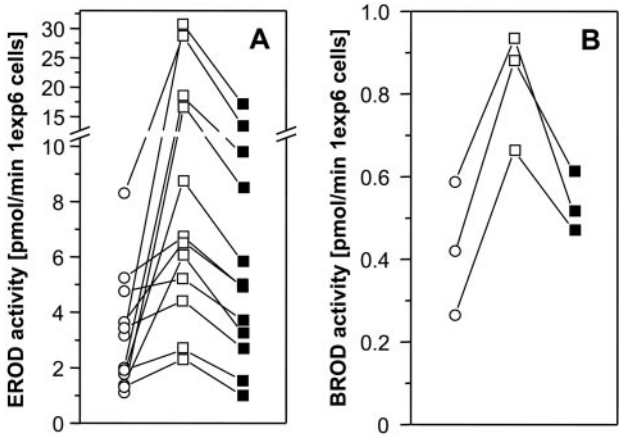

Fig. 5. Modulation of induced P450 activities of primary human hepatocytes by ALR. Twenty-four hours after plating, hepatocytes from different donors were incubated without or with 5 nM ALR for the consecutive 72 h, whereas for the last 24-h inducers, MC (2 μM) for EROD (n = 12) (A) and PB (2 mM) for BROD (n = 3) (B) were added. At 96 h of culture, activities of nontreated and noninduced cells (open circle) are compared with induced cells either without (open square) or with ALR treatment (full square). Three independent experiments are shown in triplicate.

TABLE 1

Effect of ALR on UGT activity and ratio of reduced/oxidized glutathione in primary human hepatocytes of different donors

Twenty-four hours after plating, hepatocytes were treated without or with 50 nM ALR, and UGT activity and GSH/GSSG ratio were evaluated 72 h later. UGT is expressed as nanomoles per minute and per 1 million cells, and ratios were calculated by reduced (GSH) and oxidized (GSSG) glutathione.

UGT: Control	UGT: ALR	GSH/GSSG: Control	GSH/GSSG: ALR
1.07 ± 0.07	1.07 ± 0.03	22.2 ± 4.0	20.7 ± 1.0
0.85 ± 0.07	0.84 ± 0.03	23.8 ± 3.3	40.3 ± 3.7
1.42 ± 0.06	1.44 ± 0.05	34.9 ± 7.9	20.5 ± 3.8
1.25 ± 0.01	1.16 ± 0.02	11.3 ± 2.7	10.3 ± 2.3
1.38 ± 0.11	1.29 ± 0.05		

performing activity assays. Furthermore, ALR was able to depress CYP3A4 isoenzyme expression on the basal (approximately 25%) and more intense (approximately 60%) on the Rif-induced state (Fig. 6, A and C).

Both the regulation of the constitutive and induced CYP1A2 and CYP3A4 isoenzyme repression was further analyzed by quantitative RT-PCR (Fig. 7). Hepatocytes were treated as described above for P450 protein expression analysis but with a 48-h incubation of ALR, and levels of mRNA were quantified as ratios of P450 mRNA to β-actin mRNA. ALR treatment reduced basal and MC-induced CYP1A2 mRNA expression of approximately 35%, almost to the same level as observed by protein expression studies (Figs. 6B and 7A). Levels of CYP3A4 mRNA were strongly lowered at the basal (approximately 77%) and even more at the induced (approximately 97%) (Fig. 7B) states, which showed a more intense effect of ALR on CYP3A4 mRNA than on protein expression.

ALR Induces NFκB Activity and Reduces Expression of Nuclear Receptors. It is well accepted that P450 gene expression is mediated by "orphan" nuclear receptors, and a cross-talk of endogenous mediators including growth hormones with receptor-dependent pathways was shown (Waxman, 1999). Therefore, we analyzed the impact of ALR application on mRNA expression of AhR, CAR, and PXR (Fig. 8A). Treatment of primary human hepatocytes from four different donors with 50 nM ALR did not change the expression level of AhR or PXR, whereas a significant reduction of CAR nuclear receptor expression was found. Furthermore, to shed some more light on the ALR-induced signal transduction, we determined NFκB activity a member of the NFκB transcriptional system, which is a critical signaling cascade controlling inducible gene expression. The inducible NFκB heterodimer typically consists of p65 (RelA) and p50 (NFκB-1) subunits, with RelA being the subunit conferring strong transcription activation. Incubation of human hepatocytes in vitro with the typical inducer TNF-α resulted in a high activation NFκB (Fig. 8B). Interestingly, application of ALR caused a significant NFκB activation and reached almost 40% of TNF-α induction compared with control.

Discussion

Liver regeneration is a process regulated and driven by the action of several factors such as cytokines and hepatotrophic growth factors (e.g., HGF, EGF, transforming growth factor α). Using versatile animal models and primary hepatocytes in vitro, the regeneration-promoting effect of each of these molecules was demonstrated (Blanc et al., 1992; Gomez-Lechon et al., 1996; Michalopoulos and DeFrances, 1997). Furthermore, the protein ALR was discovered to be highly expressed in regenerating livers and to significantly augment the process of hepatic healing after resection or damage. Interestingly, among all other factors, ALR was shown to exhibit its action exclusively on regenerating livers and hepatoma cells (Gandhi et al., 1999; Li et al., 2001).

In addition, the mitogenic effect of hepatotrophic growth factors such as HGF, EGF, and transforming growth factor α on human hepatocytes and their influence on hepatic drug metabolism such as P450 enzyme modulation have been discovered and characterized by several authors (Greuet et al., 1997; Donato et al., 1998; De Smet et al., 2001; Tinel et al., 2003). To our knowledge, the present study provides the first evidence of a modulating effect of the hepatotrophic factor ALR on the hepatic P450-metabolizing enzyme activities. Treatment of primary human hepatocytes with ALR led to significant alterations of different P450 isoenzyme activities

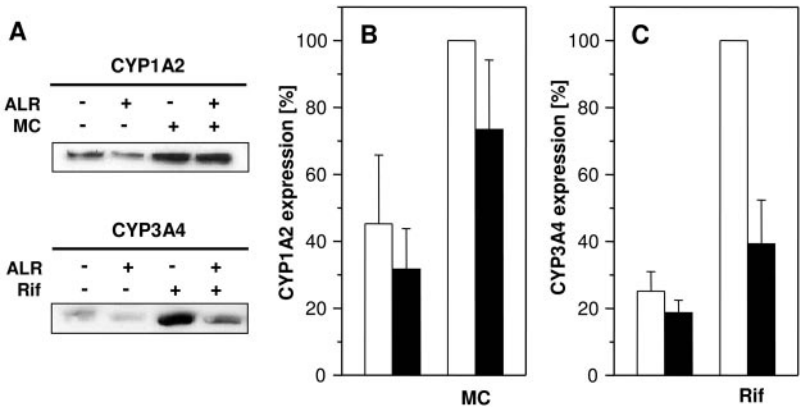

Fig. 6. Modulation of basal and induced P450 protein expression by rhALR. Twenty-four hours after plating, hepatocytes were incubated without or with 5 nM ALR for the consecutive 72 h, whereas for the last 24 h, inducers MC (2 μM) and Rif (50 μM) were added. At 96 h of culture, hepatocyte lysates (25 μg protein/lane) were analyzed by Western blot with anti-CYP1A2 and anti-CYP3A4 polyclonal antibodies. A, immunoblots for basal and induced CYP1A2 and 3A4 expression are shown. Densitometric quantification of three different individual donors was performed for CYP1A2 (B) and CYP3A4 (C). Results are normalized to induced cells without ALR treatment. Open bars, absence of ALR; solid bar, presence of ALR; three independent experiments, ± S.D.

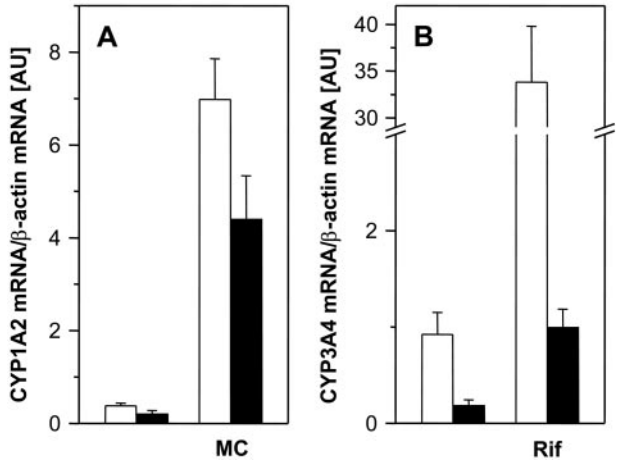

Fig. 7. Modulation of basal and induced P450 mRNA expression by ALR. Twenty-four hours after plating, hepatocytes were incubated without (open bar) or with (solid bar) 5 nM ALR for the consecutive 48 h. For the last 24 h of culture, hepatocytes were exposed to MC (2 μM) and Rif (50 μM) in the absence or presence of ALR. CYP1A2 (A) and CYP3A4 (B) mRNA expression was determined by quantitative RT-PCR analysis. Three independent experiments, ± S.D.

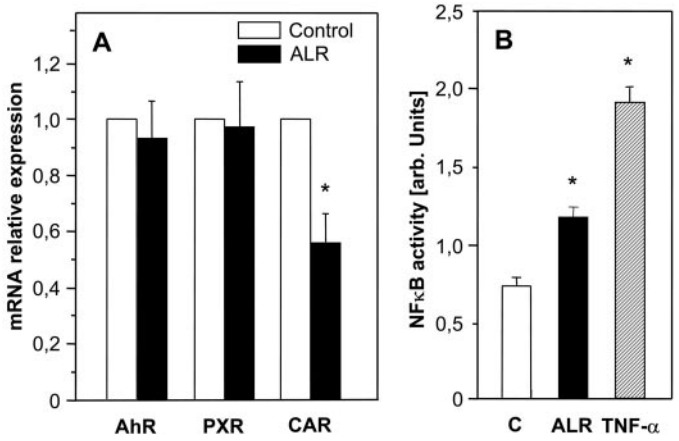

Fig. 8. Nuclear receptor expression and NFκB activation upon ALR treatment. A, 24 h after plating, hepatocytes were incubated without (open bar) or with (solid bar) 50 nM ALR for the consecutive 48 h. Expression of AhR, PXR, and CAR mRNA levels were determined by quantitative RT-PCR analysis. As an internal control, the β-actin level was measured similarly to normalize data. Data are expressed as ratio of controls. Four independent experiments, ±S.D. B, 48 h after plating, hepatocytes were incubated for 1 h with 50 nM ALR (solid bar) or 10 ng/ml TNF-α (hatched bar) or without stimuli (control, open bar). Nuclear concentration of activated NFκB was analyzed by ELISA. *, $p < 0.05$, differs from control (three independent experiments, ± S.D.).

but had no effect on phase II reactions such as UGT activity and the GSH/GSSG ratio. The repressing effect of ALR was dose-dependent for the studied CYP1A2 and CYP2A6 activities but was not restricted to a specific P450 enzyme as further demonstrated for CYP2B6 and CYP2E1 isoenzyme activities. Not only constitutive but also induced P450 enzyme activities were negatively modulated after ALR treatment. The inducers MC and Rif enhanced the expression of CYP1A2 and CYP3A4 in cultured human hepatocytes, respectively, as reported previously (Muntane-Relat et al., 1995; Greuet et al., 1997; Donato et al., 1998). ALR treatment of hepatocytes resulted in reduced P450 enzyme protein and mRNA expression, which could explain the decrease in P450 enzyme activities. Since the repressing effect of ALR was observed on different P450 enzymes, it is likely that pathways common to a wide range of P450 isoenzymes are involved. The responsible mechanisms are not known yet, but for a likely transcriptional or posttranscriptional regulation, either an activation of P450 genes or an altered rate of mRNA degradation might be necessary as proposed for the P450 enzyme regulation by EGF and HGF (Muntane-Relat et al., 1995; Donato et al., 1998).

Furthermore, several authors have suggested that the production of nitric oxide (NO) leads to down-regulation of P450 enzyme activities after inflammation or tissue injury (Levitchi et al., 2004). Cytokines and potentially growth factors may induce NO synthase expression and, therefore, may be responsible for high NO levels found in regenerative livers. NO was shown to inactivate P450 enzyme activity by binding to the P450 protein (Levitchi et al., 2004). In this study, we did not detect an increased NO release into the culture medium during ALR treatment. In addition, supplementation with N^{ω}-nitro-L-arginine methyl ester, a specific inhibitor of NO synthase, did not prevent P450 repression (data not shown). Therefore, negative modulation of P450 enzyme activity is probably not mediated by NO as was concluded for EGF and HGF treatment of human hepatocytes (Muntane-Relat et al., 1995; Donato et al., 1998).

Hepatocytes in a normal healthy liver are in a quiescent state but get easily activated to enter the cell cycle for proliferation after hepatectomy (Michalopoulos and DeFrances, 1997). It was already shown by several authors that after cell isolation, primary human hepatocytes in culture leave the G_0 phase to enter the cell cycle and progress to the late G_1 phase indicated by expression of various immediate-early oncogenes including *c-myc* and *c-fos*. This "priming" step ends at

the so-called restriction point in the G_1 phase, rendering the hepatocytes competent to respond to mitotic factors, and crossing from G_1 to S phase only occurs in the presence of growth factors like EGF and HGF (Blanc et al., 1992; Gomez-Lechon et al., 1996; Greuet et al., 1997). Both the activation of hepatocyte proliferation and the P450 gene repression upon growth factor treatment was shown to be dependent on culture time (lag period after cell plating) and duration of application. HGF supplementation led to CYP1A2 reduction when hepatocytes were treated for 72 to 120 h coincident with a maximum in DNA synthesis but presumably after cells crossed the restriction point (Donato et al., 1998). On the other hand, EGF exerted its proliferative and P450 gene-repressing effect in the priming period of hepatocytes as described earlier (Greuet et al., 1997). Furthermore, the authors argued that the loss of cell-cell contacts observed in subconfluent cultures has a major influence on reduced P450 expression. This impact could be minimized in our study applying confluent cultures (see *Material and Methods*). The maximum effect of ALR seen at 24 to 72 h is in coincidence with action of growth factors in the priming period; therefore, it might be assumed that activated pathways and their related signals for proliferation are involved in P450 gene expression.

Further evidence for this hypothesis comes from a recent report investigating the decreasing effect of sera from humans with inflammatory reactions on CYP1A1/2 activity. Using different inhibitors of signaling pathways, they could demonstrate that the activation of protein tyrosine kinases and p42/44 mitogen kinase are involved in P450 repression (Levitchi et al., 2004). On the other hand, it was reported that ALR upon binding to its own specific receptor (Wang et al., 1999) acts through stimulation of the tyrosine phosphorylation of the epidermal growth factor receptor and activates the MAPK cascade indicated by phosphorylated MAPK kinase and extracellular signal-regulated kinase (p42/44 mitogen kinase) (Li et al., 2000). Therefore, we assume that the activation of extracellular signal-regulated kinase by ALR might be at least in part involved in the P450 repression but should be verified in the future.

Another member of the MAPK family, p38 MAPK, activated by various stimuli has been discussed to regulate hepatic metabolism like phase II reactions (Yu et al., 2000) but failed to be responsible for P450 modulation by IL-6 (Jover et al., 2002) or phenobarbital (Joannard et al., 2005). Additionally, it was reported that in some cases such as CYP2E1, the P450 activity is regulated at the protein expression levels through protein stabilization or degradation (Danielson, 2002) or at the mRNA level through increased translation (Abdelmegeed et al., 2005). In the latter case, an involvement of p38 MAPK could be excluded, although a role of p38 MAPK for stabilizing P450 mRNA was discussed (Joannard et al., 2005). In our study, we found ALR with no effect on phase II reactions, and further studies about a modulating effect of p38 MAPK have to be undertaken.

Various mechanisms have been proposed to explain the down-regulating effect of cytokines and growth factors on P450 genes. It was reported that TNF-α represses CYP1A1 via redox regulation of nuclear factor 1 (Morel and Barouki, 1998), EGF represses CYP2C11 and CYP3A via overexpression of c-myc and decreased C/EBP binding to DNA (Tinel et al., 2003), interleukin 1 inhibits CYP2C11 transcription via NFκB binding to the promoter (Iber et al., 2000), and IL-6 inhibits CYP3A4 via induction of C/EBPβ-LIP, a negative competitor of C/EBPα (Jover et al., 2002). Previously, it was shown that IL-6 can also repress P450 (CYP3A4, CYP2B6) via down-regulation of PXR and CAR (Pascussi et al., 2000), members of the nuclear receptor family proven to regulate P450 gene expression (Waxman, 1999; Honkakoski and Negishi, 2000). Additionally, it was reported that interleukin 1β decreases CYP3A4 and CYP2B6 expression by interaction of activated NFκB with the glucocorticoid receptor, which mediates CAR transactivation (Assenat et al., 2004). ALR induces NFκB activity as well as reduces CAR expression in human hepatocytes; therefore, we may assume that, at least in part, NFκB activation is responsible for reduced CAR levels and, therefore, altered P450 gene expression. Furthermore, it was shown that NFκB interacts with the Ah receptor (aryl hydrocarbon) and that this interaction is involved in suppression of CYP1A1/2 gene expression (Tian et al., 2002) (see summary in Supplemental Fig. 1).

Reduction of P450 gene expression in association with liver disease and liver regeneration were widely studied, demonstrating impaired drug biotransformation and detoxication capacity are mainly mediated by P450 and regulated by a variety of molecules such as cytokines and growth factors (Morgan, 2001; Villeneuve and Pichette, 2004). Increased ALR serum levels were detected for various types of acute liver disease (Tanigawa et al., 2000); in addition, we could find increased expression of ALR in livers from patients with cirrhosis and hepatocellular and cholangiocellular carcinoma (Thasler et al., 2005). Therefore, in conclusion, we showed that ALR not only acts as augmenter of cell proliferation but also concomitantly reduces P450 enzyme activities by gene repression at least in part mediated through NFκB activation and CAR down-regulation. Furthermore, these findings suggest that the hepatotrophic factor ALR in addition to other mitogenic factors may activate intracellular signaling pathways that cross-talk with the receptor-dependent pathways contributing to the reduction of P450 activities during liver regeneration and disease.

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