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Interleukin-1β, interleukin-6, tumour necrosis factor-α and interferon-γ released by a viral infection and an aseptic inflammation reduce CYP1A1, 1A2 and 3A6 expression in rabbit hepatocytes

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Abstract

Inflammation reduces activity and expression of hepatic cytochrome *P*450 (P450) and therefore diminishes drug biotransformation. This study aimed to identify the serum mediators triggered by a viral infection and an aseptic inflammation that downregulate P450 isoforms. Incubation of hepatocytes with serum from rabbits with a turpentine-induced inflammation or humans with a viral infection decreased the amount of cytochrome 1A1 (CYP1A1), 1A2 and 3A6 mRNA and apoproteins. By serum fractionation and immuno-neutralization, we showed that in the aseptic inflammation, interleukin-6 and, to a lesser degree, interleukin-1β are involved in the downregulation of all three isoforms. In serum from humans with a viral infection, interleukin-1β, interleukin-6, interferon-γ and tumour necrosis factor-α contribute to the downregulation of P450 isoforms. CYP1A1 and 1A2 are regulated by serum mediators at the transcriptional level, while the expression of CYP3A6 appears to be under the control of pre- and posttranscriptional mechanisms.

Keywords: Cytochrome *P*450; Downregulation; Inflammation; Serum mediator; Cytokine

1. Introduction

Inflammation and infection can have a profound impact on hepatic drug metabolism. It has been known for more than three decades that activation of host-defence responses reduces the activity of cytochrome P450 monooxygenases (P450) (Morgan, 1997). This phenomenon is of utmost importance since P450 enzymes play a crucial role in the metabolism of a variety of endogenous and exogenous compounds. Changes in the expression of P450 will affect the rate of drug biotransformation and modify drug plasma and tissue concentrations, with consequences on therapeutic effect and toxicity (Chang et al., 1978; Monshouwer and Witkamp, 2000).

In animal models, stimulation of the acute phase response by agents such as viruses, bacteria, endotoxins, turpentine and interferon decrease the activity and amount of P450. It has been postulated that the reduction in P450 expression is mediated by cytokines because in vivo, the administration of recombinant cytokines such as interleukin-1, interleukin-2, interleukin-6, interferon-γ and tumour necrosis factor-α diminish P450-mediated drug metabolism and downregulate selective P450 isoforms. In vitro, these cytokines depress CYP1A1/A2, 2C11, 2E1 and CYP3A subfamily (Abdel-Razzak et al., 1993; Muntané-Relat et al., 1995; Chen et al., 1995; Siewert et al., 2000; Delaporte et al., 1993).

In vivo, the downregulation of CYP1A1, 1A2 and 3A6 caused by a turpentine-induced inflammatory reaction is preceded by the reduction in the activity of these isoforms (Barakat et al., 2001). In vitro, incubation of hepatocytes of rabbits with serum from humans with an upper respiratory viral infection or serum from rabbits with a turpentine-induced inflammatory reaction for 4 h decreases P450 activity without affecting the expression of P450 isoforms (El-Kadi et al., 1997). The decrease in P450 activity by serum from rabbits with a turpentine-induced inflammatory reaction is almost totally dependent upon interleukin-6; in contrast, in serum from humans with a viral infection, interferon-γ, interleukin-1β and interleukin-6 contribute to

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the reduction of P450 activity (Bleau et al., 2000). Incubation of serum from rabbits with a turpentine-induced inflammatory reaction and serum from humans with a respiratory viral infection with hepatocytes for 24 and 48 h downregulate several isoforms of P450 (Bleau et al., 2001). The present study aimed to determine the nature of the mediators in serum from humans with an upper respiratory viral infection and in serum from rabbits with a turpentine-induced inflammatory reaction that downregulate P450 isoforms.

2. Materials and methods

2.1. Hepatocyte isolation and culture conditions

Livers from male New Zealand rabbits (2–2.2 kg) obtained from Ferme Charles Rivers (St-Constant, Quebec, Canada) were perfused in situ with collagenase using the two-step liver perfusion method described by Seglen (1976), with minor modifications (El-Kadi et al., 1997). Harvested cells were centrifuged on isodensity Percoll to isolate viable liver cells (90% viability as assessed by trypan blue exclusion). Hepatocytes (3×10^6 in 3 ml of William's medium E supplemented with 10% calf serum) were plated in 12-well plastic culture plates (Falcon, Becton Dickinson Labware, Rutherford, NJ) coated with type I rat tail collagen. Cell culture was conducted under sterile conditions and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air. The medium was changed 2 h after plating (prior to the addition of serum or HPLC fractions), and cells were incubated for 24 and 48 h. At the end of the incubation period, liver cells were washed in 3 ml of PBS and flash-frozen into liquid nitrogen. Samples were kept at −80 °C until analysis. All the experiments were conducted according to the Canadian Council on Animal Care guidelines for use of laboratory animals.

2.2. Preparation of rabbit and human serum

A local inflammatory reaction was induced by the subcutaneous injection of 5 ml of turpentine (Recochem, Montréal, Québec) distributed into four distinct sites of the back of the rabbits. Forty-eight hours later, blood (20 ml) was withdrawn from the central artery of an ear of the animals. Human blood (10 ml) was withdrawn from volunteers, either healthy (n=7) or presenting an inflammatory reaction secondary to an upper respiratory viral infection (n=7), at the apex of clinical symptomatology, i.e., 24–48 h after the onset of clinical symptomatology (fever, rhinorrhea, sneezing, nasal congestion, sore throat, cough and systemic signs of malaise). Blood samples were allowed to clot at room temperature for 2 h, centrifuged at 2500 rpm for 5 min, and the serum was decanted and stored frozen at −80 °C in 1-ml aliquots until use. Human sampling protocol was approved by the Comité d'Éthique de la Recherché de la Faculté de Médecine of the University of Montréal (CERFM 28(00) 4#78).

Aliquots of 200 μl of serum from control rabbits and rabbits with a turpentine-induced inflammatory reaction, as well as serum from healthy human volunteers and from humans with respiratory viral infection, were incubated for 24 and 48 h with control hepatocytes, and the effect on P450 was assessed by measuring CYP1A1, CYP1A2 and CYP3A6 apoproteins and the mRNA coding for these isoforms.

2.3. Fractionation of serum proteins

To characterise the proteins in serum responsible for the downregulation of P450 isoforms, the serum was fractionated according to the Mr of the proteins by size exclusion high-performance liquid chromatography (HPLC) on a Superose 12 HR column (Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) as described elsewhere (Bleau et al., 2000). Briefly, column pressure was maintained between 8 and 10 bar, flow rate was set at 0.3 ml/min with a LKB 2150 HPLC pump (Bromma, Sweden) and absorbance was measured at 280 nm with a Waters 490E spectrophotometric detector (Millipore, Milford, MA). The eluant buffer contained 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM HEPES, 25 mM EGTA and 5.5 mM glucose; this solution was adjusted to pH 7.4 and filtered through a 0.22-μm membrane. To calculate the Mr of the serum proteins contained in each HPLC fraction, a calibration curve was established by injecting 300 μl of buffer containing a mixture of six standard proteins (100 μg/ml): L-glutamic dehydrogenase (55.6 kDa), aldolase (39.2 kDa), triosephosphate isomerase (26.6 kDa), trypsin inhibitor (26.6 kDa), cytochrome c (12.5 kDa) and aprotinin (6.5 kDa). Aliquots of 300 μl of each serum were injected into the column, and fractions of 1.2 ml were collected with a fraction collector (LKB 2211 Superrac). The fractions recovered corresponded to proteins with Mr included in the range of 9–14, 15–23, 24–31, 32–44, 45–64 and 65–94 kDa. Since the column could not separate proteins in the high Mr region, the proteins contained in the first fraction were designated as Mr>95 kDa.

In order to increase sensitivity, collected fractions were concentrated on Microsep 3 K membranes (Pall Filtron, Northborough, MA), which retain proteins of more than 3 kDa, to reach the final volume of 300 μl, as the original serum. This provided protein concentrations equivalent to those in whole serum and made possible the comparison of the respective activities.

2.4. Immuno-neutralisation of cytokines

In serum fractions, the proteins decreasing CYP1A1, CYP1A2 and CYP3A6 expression were identified by immuno-neutralization. One polyclonal and four monoclonal antibodies against cytokines were used to neutralise P450 downregulation induced by serum and HPLC fractions. To immuno-neutralise mediators in rabbit serum, the

following antibodies were used: a goat anti-rabbit interleukin-1β antibody, monoclonal antibodies against human interleukin-6 and human interferon-γ and a sheep anti-human tumour necrosis factor-α antibody α. The three antibodies against human cytokines were used to neutralise the homologous rabbit cytokines because of the known cross-reactivity of these antibodies (Huang et al., 1997; Muscettola et al., 1995; Herbertson et al., 1995). The same antibodies against human cytokines as well as a monoclonal anti-human interleukin-1β were used to neutralise mediators in human serum and fractions. An irrelevant monoclonal antibody (immunoglobulin G to *Pseudomonas aeruginosa*) served as control. Aliquots of 2 μg of each antibody were added to 200 μl of the concentrated HPLC fractions able to downregulate P450 and preincubated at 37 °C for 1 h. The combination of the four antibodies was incubated only with serum from rabbits with a turpentine-induced inflammatory reaction and serum from humans with a respiratory viral infection. Antibody-treated sera or HPLC fractions were added to hepatocytes at the beginning of the incubation period.

To confirm the ability of cytokines to downregulate P450 isoforms, recombinant human interleukin-1β, interleukin-6, tumour necrosis factor-α and interferon-γ (2.5–10 ng/ml) were incubated for 48 h with hepatocytes.

2.5. Measurement of cytokines in serum

Interferon-γ was measured by a sandwich solid-phase RIA using two anti-interferon-γ mouse monoclonal antibody (mAb) (clone 42.25) to coat the solid phase and ^{125}I-labeled anti-interferon-γ mAb (clone KM48) as detecting probe. Tumour necrosis factor-α was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using mAb to human tumour necrosis factor-α (cloned T144.B). The sensitivity of the assay was 30 pg ml^{-1} for interferon-γ and 45 pg/ml for tumour necrosis factor-α. Interferon-γ and tumour necrosis factor-α were measured in duplicate in the laboratory of Drs. G. Delespesse and M. Sarfati as described elsewhere (Armant et al., 1994). Interleukin-6 and interleukin-1β were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN).

2.6. Measure of CYP1A1, 1A2 and 3A6 protein expression

Protein content in hepatocytes was measured by the method described by Lowry et al. (1951) and aliquots of 4 μg of proteins were electrophoresed on sodium dodecyl sulfate–polyacrylamide (7.5%) gels under nonreducing conditions. Separated proteins were electrophoretically transferred to a nitrocellulose membrane using a semi-dry transfer process (Bio-Rad, Hercules, CA, USA). Bands were immunologically detected using a polyclonal anti-rabbit CYP1A1–1A2 (Oxford Biochemical Research, Oxford, MI, USA) diluted 1:100 in 5% nonfat milk in TBS/0.1% Tween 20 and visualized with an alkaline phosphatase-conjugated secondary goat antibody using nitro blue tetrazolium as the substrate (Kruger, 1994). CYP3A6 protein was detected with a monoclonal anti-rat CYP3A1, with cross-reactivity to rabbit's CYP3A6 (Oxford Biochemical Research), diluted to 1:100 with 5% nonfat milk in TBS/0.1% Tween 20 using a horseradish peroxidase-conjugated secondary antibody. Chemiluminescence was visualised by autoradiography (Thorpe et al., 1985). In each gel, 50 μg of proteins extracted from the same control hepatocytes, with constant amounts of CYP1A1, 1A2 and 3A6, were used as reference proteins. Band intensities were measured with a software Un-Scan-It-Gel (Silk Scientific, Orem, UT). Data are presented in arbitrary units as the ratio of the densitometric values of the bands for each sample over that of the reference protein.

2.7. Preparation of cDNA probes

The complete cDNA sequences for rabbit *CYP1A1* and *1A2* (corresponding to pLM-6 and pLM-4 gene, respectively) were available as inserts in pUC19, cloned in the *Lac Z* region between the *EcoR*I (396 bp) and PstI (440 bp) sites (Dalet et al., 1998). The cDNA sequence for *CYP3A6* (p-4.1 gene) was an insert in pBR322, cloned at the Pst-1 site (3609 bp). A small cDNA fragment for *CYP1A1* was isolated by the digestion of the recombinant plasmid with the enzymes Pst-1 and Not-I, which generated a 261-bp fragment. Similarly, a 329-bp fragment for *CYP1A2* was generated by digestion of the construct with Pst-1 and Sac-I, and the 324-bp fragment for *CYP3A6* was excised using Sal-I and Cla-I. Fragments were purified using the NucleoTrap extraction kit (Clontech, Palo Alto, CA) and inserted into the pBluescriptII plasmid (Stratagene, La Jolla, CA) containing sites for RNA polymerase (for T7 and T3 promotors). Plasmids were first dephosphorylated with alkaline phophatase and ligated to the DNA fragment using a T4 DNA ligase (Life Technologies, Rockville, MD). Bacteria (*Escherichia coli*) were transfected with the constructs by heat shock and seeded on petri dishes containing LB culture medium without ampicillin. Transfected bacteria were incubated overnight in 5 ml of LB medium containing ampicillin with vigorous agitation. The recombinant clones were analysed by the Miniprep method and digestion with restriction enzymes to generate the needed fragments.

The three cDNA probes were sequenced in both directions by the Sanger method using the Sequenase 2.0 kit (United States Biochemicals, Cleveland, OH). Sp6 and T7 primers were used, and the cDNA fragments were sequenced twice. The results of sequencing demonstrated 100% identity of each fragment with its sequence in Genbank.

2.8. Northern blot analysis

Total RNA was isolated using 1 ml of Trizol Reagent (Life Technologies) per $5–10\times10^6$ cells. RNA concentration was measured spectrophotometrically at the absorbance of 260

nm (with an $A_{260/280}$ ratio ≈ 2). Total RNA samples were denatured by heating at 60 °C for 10 min in buffer containing 42% deionised formamide, 30 mM 4-morpholinepropanesulfonic acid and 8.5% formaldehyde. Thirty micrograms of RNA were then separated by electrophoresis in a denaturing 1% agarose–1.7% formaldehyde gel submerged in a buffer containing 20 mM 4-morpholinepropanesulfonic acid, 8 mM sodium acetate and 1 mM EDTA, pH 7.2. Isolated RNA was transferred to a nylon membrane (Qiabrane, Qiagen) by capillary blotting with a solution of 1.2 M NaCl and 0.15 M sodium citrate, pH 7.0. RNA was fixed to the membrane by exposure to UV light. Membranes were prehybridised for 30 min at 42 °C using the ULTRAhyb hybridisation buffer (Amicon, Austin, TX, USA). The cDNA probes for *CYP1A1-A2-3A6* and 18S were labelled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) using Klenow fragment according to the oligo-priming method of the Oligo-labelling kit (Amersham Pharmacia Biotech, Piscataway NJ). Hybridisation was performed at 42 °C for 24 h with the radiolabeled cDNA probe and the blots washed as recommended by the manufacturer. Membranes were subjected to autoradiography film Biomax with Biomax TranScreen-HE intensifying screens (Kodak) at −80 °C during 24 h. Data are presented as the ratio of the densitometric value of the isoforms of the P450 over that of the 18S.

Two forms of *CYP3A6* mRNA are expressed in rabbit liver, one with 1.85 kb and the other with 1.7 kb. These forms differ by the length of their 3′ untranslated region, and code for the same protein (Dalet et al., 1998). Both forms were separated and quantified simultaneously.

2.9. Drugs and chemicals

The Percoll gradient, William's medium E, calf serum, type I rat tail collagen, NaCl, KCl, KH_2PO_4, HEPES, EGTA and glucose were purchased from Sigma (St. Louis, MO), insulin from Boehringer Mannheim Biochemica (Mannheim, Germany), and L-glutamic dehydrogenase, aldolase, triosephosphate isomerase, trypsin inhibitor, cytochrome *c* and aprotinine from Pharmacia Biotech. The polyclonal anti-rabbit CYP1A1 and the monoclonal anti-rat CYP3A1 was obtained from Oxford Biochemical Research, the goat anti-rabbit interleukin-1β from Cedar Lane (Hornby, Ontario, Canada), the monoclonal antibodies against human interleukin-6 and human interferon-γ from R&D Systems. The sheep anti-human tumour necrosis factor-α antibody was graciously given by Protherics.

2.10. Statistical analysis

All data are reported as means±S.E. Comparisons between two treatments were carried out using a paired *t*-test and between treatment groups using a one-way analyses of variance (ANOVA) followed by Newman–Keuls post hoc test. The differences were considered statistically significant when the probability was $P<0.05$.

3. Results

3.1. In vivo effect of a turpentine-induced inflammatory reaction on the expression of hepatic P450 proteins and mRNA content

Compared with the amount of apoproteins in control hepatocytes, 48 h after the induction of the turpentine-induced inflammatory reaction, protein expression of CYP1A1 was reduced by 54%, that of CYP1A2 by 46%, and that of CYP3A6 to almost undetectable levels ($P<0.05$) ($n=3$). Concomitant to the downregulation of the apoproteins, the mRNAs encoding for *CYP1A1* and *CYP1A2* were reduced by 63% and 66%, respectively, and the two forms of *CYP3A6* mRNA were diminished by 62% and 63% ($P<0.05$) ($n=3$) (Fig. 1).

3.2. Effect of serum from rabbits with a turpentine-induced inflammatory reaction and HPLC fractions on P450 protein expression and mRNA content in control hepatocytes

Incubation of serum from control rabbits and the corresponding HPLC fractions with hepatocytes did not change the protein expression of P450 isoforms. Addition of serum from rabbits with a turpentine-induced inflammatory reaction to hepatocytes for 24 h did not modify CYP1A1 and CYP1A2 proteins expression; however, it decreased CYP3A6 by 20% ($P<0.05$) ($n=6$) (data not shown). When the incubation was prolonged to 48 h, CYP1A1 was reduced by 26%, CYP1A2 by 25% and CYP3A6 by 27% ($P<0.05$) ($n=7$) (Table 1). Incubation of the HPLC fractions of serum from rabbits with an inflammatory reaction with hepatocytes for 24 h showed that only the 24–31- and 15–23-kDa fractions reduced CYP3A6 apoprotein by 32% and 19%, respectively (data not shown). When the HPLC fractions of serum from rabbits with an inflammatory reaction were incubated with hepatocytes for

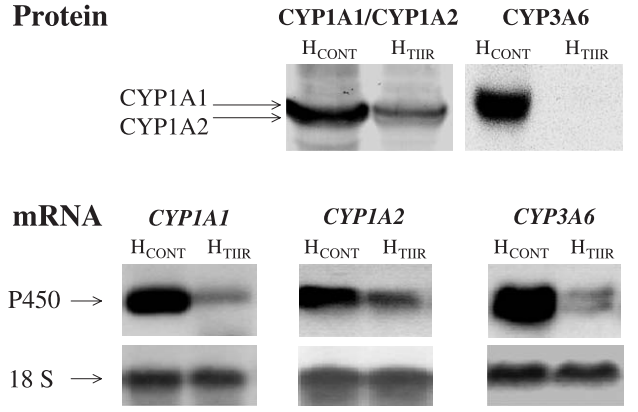

Fig. 1. Representative bands illustrating the expression of CYP1A1, 1A2 and 3A6 proteins and the content of the mRNA encoding for these proteins in hepatocytes from control rabbits (H_{CONT}) and from rabbits with a turpentine-induced inflammatory reaction (H_{TIIR}) 48 h after the injection of turpentine.

Table 1
Effect of serum from control rabbits (RS_{CONT}), serum from rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}) and of HPLC fractions of RS_{TIIR} on the expression of CYP1A1 protein (n=7) and *CYP1A1* mRNA content (n=3), CYP1A2 protein (n=4) and *CYP1A2* mRNA content (n=3), and CYP3A6 protein (n=4) and *CYP3A6* mRNA content (n=3) in hepatocytes from control rabbits (H_{CONT}) after 48 h of incubation

	RS_{CONT}	RS_{TIIR}	<95	65–94	45–64	32–44	24–31	15–23	9–14
CYP1A1	0.57±0.07	0.42±0.07[a]	0.51±0.08	0.47±0.07	0.49±0.07	0.49±0.08	0.40±0.06[a]	0.38±0.06[a]	0.54±0.11
CYP1A1 mRNA	0.44±0.11	0.26±0.07[a]	0.48±0.14	0.38±0.15	0.45±0.15	0.48±0.13	0.23±0.06	0.41±0.13	0.52±0.11
CYP1A2	0.60±0.05	0.45±0.05[a]	0.56±0.05	0.56±0.04	0.60±0.05	0.55±0.05	0.46±0.06[a]	0.45±0.05[a]	0.55±0.05
CYP1A2 mRNA	0.63±0.11	0.36±0.08[a]	0.55±0.20	0.45±0.20	0.53±0.22	0.55±0.22	0.33±0.05	0.41±0.11	0.59±0.22
CYP3A6	0.43±0.05	0.31±0.05[a]	0.42±0.07	0.42±0.04	0.42±0.05	0.41±0.03	0.30±0.06[a]	0.29±0.07[a]	0.43±0.06
CYP3A6 mRNA	0.52±0.13	0.33±0.06[a]	0.39±0.15	0.47±0.15	0.45±0.16	0.36±0.17	0.26±0.16	0.30±0.17	0.44±0.20
CYP3A6 mRNA	0.54±0.08	0.36±0.03[a]	0.34±0.02	0.37±0.04	0.39±0.03	0.42±0.08	0.23±0.07	0.26±0.03	0.40±0.03

Values are mean±S.E.
[a] $P<0.05$ compared with RS_{CONT}.

48 h, the expression of CYP1A1, CYP1A2 and CYP3A6 were decreased by around 25% by both 24–31- and 15–23-kDa fractions ($P<0.05$) (n=4) (Table 1).

Compared with serum from control rabbits, incubation of hepatocytes with serum from rabbits with a turpentine-induced inflammatory reaction for 24 h did not modify the levels of mRNA for any of the three isoforms of P450 tested. However, after 48 h of incubation, serum from rabbits with an inflammatory reaction downregulated mRNA content for *CYP1A1* by 42%, *CYP1A2* by 38% and the two forms of *CYP3A6* by 39% and 35% ($P<0.05$) (n=6) (Table 1).

As seen with whole serum, 24 h incubation of hepatocytes with individual HPLC fractions of serum from rabbits with a turpentine-induced inflammatory reaction did not change the mRNA content for any of the three isoforms. When the incubation was prolonged to 48 h, the fractions containing proteins with a Mr of 15–23 and 24–31 kDa tended ($P>0.05$) to diminish *CYP1A1*, *CYP1A2* and the two forms of *CYP3A6* mRNA by 30–60% (n=3) (Table 1). The fact that both HPLC fractions elicited a nonsignificant effect on mRNA content but the whole serum decreased it suggests that the amount of mediators in each fraction was insufficient to reduce significantly mRNA content even if the repercussion on protein expression was evident.

3.3. Identification of the mediators in serum from rabbits with a turpentine-induced inflammatory reaction responsible for the downregulation of P450 isoforms

The former results strongly suggest that the effect of serum from rabbits with a turpentine-induced inflammatory reaction results from the effect elicited by the mediators comprised in fractions 15–23 and 24–31 kDa; therefore, after fractionation of the sera, the 15–23- and 24–31-kDa fractions were combined, tested for their ability to reduce the amount of P450 isoforms and used for the immunoneutralisation experiments.

Compared with serum from control rabbits, incubation of hepatocytes with serum from rabbits with a turpentine-induced inflammatory reaction and the 15–31-kDa fraction for 48 h reduced CYP1A1, 1A2 and 3A6 protein expression by approximately 25% ($P<0.05$) (n=4) (Fig. 2). The effect of the 15–31-kDa fraction on CYP1A1 was partially prevented by the anti-interleukin-6 antibody. The downregulation of CYP1A2 and 3A6 by the 15–31-kDa fraction was hindered by the anti-interleukin-1β and anti-interleukin-6 antibodies (Fig. 2). Anti-interferon-γ and anti-tumour necrosis factor-α antibodies did not reverse the effect of the 15–31-kDa fraction on any of the apoproteins. When all four antibodies were added to serum from rabbits with a turpentine-induced inflammatory reaction and incubated with hepatocytes, the serum did not decrease CYP1A1, 1A2 and 3A6 expression (Fig. 2).

3.4. Effect of serum from humans with an upper respiratory viral infection and HPLC fractions on P450 protein expression and mRNA content in control hepatocytes

Incubation of hepatocytes with serum from healthy volunteers and its HPLC fractions did not modify P450 expression (data not shown). Compared with serum from healthy volunteers, after 24 h of incubation, serum from humans with an upper respiratory viral infection reduced the expression of CYP3A6 by 24%. Incubation of hepatocytes with serum from humans with a viral infection and the 15–23-kDa fraction for 48 h diminished the amount CYP1A1, CYP1A2 and CYP3A6 ($P<0.05$, n=7 for serum and n=4 for HPLC fractions) (Table 2).

Compared with serum from healthy volunteers, incubation of serum from humans with an upper respiratory viral infection with hepatocytes for 24 h downregulated the mRNA of *CYP1A1* by 33%, that of *CYP1A2* by 32% and the two forms of *CYP3A6* mRNA by 32% and 20% ($P<0.05$; n=6) (data not shown). On the other hand, incubation for 48 h reduced mRNA of *CYP1A1* by 56%, of *CYP1A2* by 58% and that of the two forms of *CYP3A6* by 33% and 45% ($P<0.05$; n=6) (Table 2).

Of the HPLC fractions tested, the 15–23-kDa fraction incubated with hepatocytes for 24 h (data not shown) and for 48 h downregulated *CYP1A1* mRNA by 35% and 58%, respectively ($P<0.05$; n=3) (Table 2). Following 48 h incubation, the 15–23- and 24–31-kDa fractions downregulated *CYP1A2* mRNA by 60% and 56%, respectively ($P<0.05$; n=3). After 48 h incubation of the 15–23-kDa fraction of

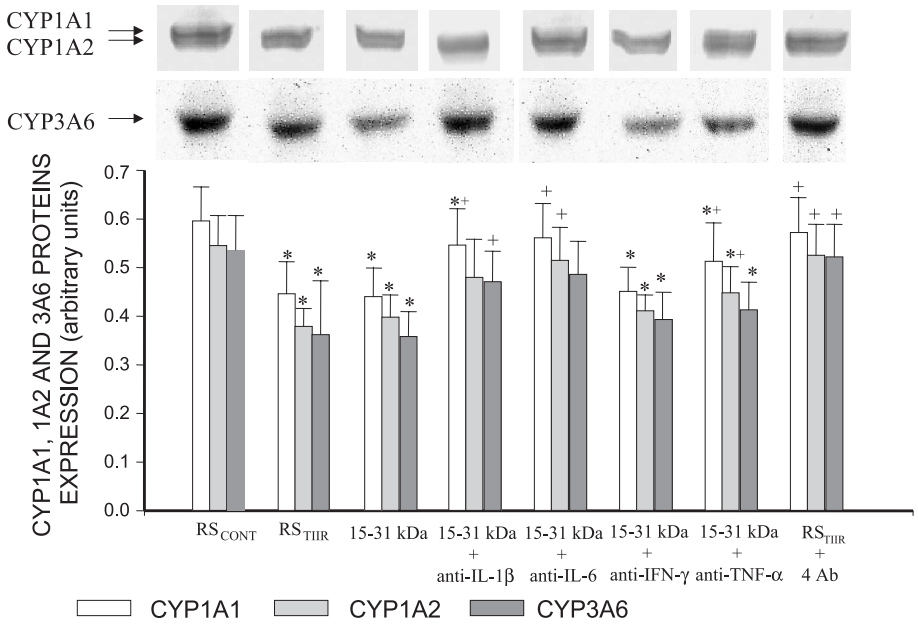

Fig. 2. Effect of anti-cytokine antibodies (Ab) on the ability of the 15–31-kDa HPLC fraction of serum from rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}) to reduce the expression of CYP1A1, 1A2 and 3A6 proteins after 48 h of incubation with hepatocytes from control rabbits (H_{CONT}). The upper panel of the figure illustrates representative gels. RS_{CONT}: serum from control rabbits. Values on the abscissa correspond to Mr of the proteins contained in each HPLC fraction. Data are mean±S.E. of the ratio of the sample over the control. *P<0.05 compared with RS_{CONT} (n=4) or 15–31 kDa fraction (n=4), respectively.

serum from humans with an upper respiratory viral infection with the hepatocytes, the contents of the 1.7- and 1.8-kb *CYP3A6* mRNA were not different from control (n=3) (data not shown).

3.5. Identification of the mediators in serum from humans with an upper respiratory viral infection responsible for the downregulation of P450

The mediators of serum from humans with an upper respiratory viral infection were assessed in the 15–31-kDa combined fraction. Compared to serum from healthy volunteers, the addition serum from humans with an upper respiratory viral infection and the 15–31-kDa fraction to hepatocytes reduced CYP1A1, CYP1A2 and 3A6 protein expression (P<0.05) following 48 h of incubation (Fig. 3). Preincubation of the 15–31-kDa fraction with anti-tumour necrosis factor-α and anti-interferon-γ antibodies partially prevented the downregulation of CYP1A1. Anti-interleukin-6, anti-interferon-γ and anti-tumour necrosis factor-α partly hindered the diminution in expression of CYP1A2. All four antibodies when incubated with the 15–31-kDa fraction prevented to some extent the decrease in CYP3A6 apoprotein. Combination of the four antibodies incubated at the same time with serum from humans with an upper respiratory viral infection totally impeded the decrease in CYP1A1, CYP1A2 and 3A6 protein expression, i.e., the amounts were equal to those observed in hepatocytes incubated with serum from healthy volunteers (Fig. 3).

3.6. Presence of cytokines in human serum and repressive effect of recombinant human cytokines

In serum from healthy volunteers, cytokines concentrations were below the detection limit of the methods used. Samples of serum from five subjects with an upper respi-

Table 2
Effect of human serum from controls (HS_{CONT}) and subjects with an upper respiratory tract viral infection reaction (HS_{URVI}) and of HPLC fractions of HS_{URVI} on the expression of CYP1A1 protein (n=4) and *CYP1A1* mRNA content (n=3), CYP1A2 protein (n=4) and *CYP1A2* mRNA content (n=3), and CYP3A6 protein (n=4) in hepatocytes from control rabbits (H_{CONT}) after 48 h of incubation

	HS_{CONT}	HS_{URVI}	<95	65–94	45–64	32–44	24–31	15–23	9–14
CYP1A1	0.53±0.08	0.33±0.08[a]	0.48±0.08	0.53±0.10	0.48±0.08	0.48±0.09	0.45±0.11	0.36±0.06[a]	0.49±0.11
CYP1A1 mRNA	0.30±0.11	0.17±0.05[a]	0.23±0.04	0.31±0.09	0.32±0.08	0.27±0.08	0.23±0.08	0.13±0.03[a]	0.21±0.07
CYP1A2	0.73±0.06	0.32±0.13[a]	0.68±0.06	0.67±0.08	0.66±0.08	0.72±0.08	0.58±0.06[a]	0.49±0.05[a]	0.57±0.05
CYP1A2 mRNA	0.60±0.14	0.25±0.09[a]	0.44±0.07	0.46±0.06	0.53±0.06	0.47±0.08	0.32±0.02[a]	0.23±0.01[a]	0.47±0.06
CYP3A6	0.53±0.08	0.33±0.08[a]	0.51±0.10	0.61±0.11	0.52±0.08	0.56±0.07	0.45±0.06	0.34±0.08[a]	0.42±0.07

Values are mean±S.E.
[a] P<0.05 compared with HS_{CONT}.

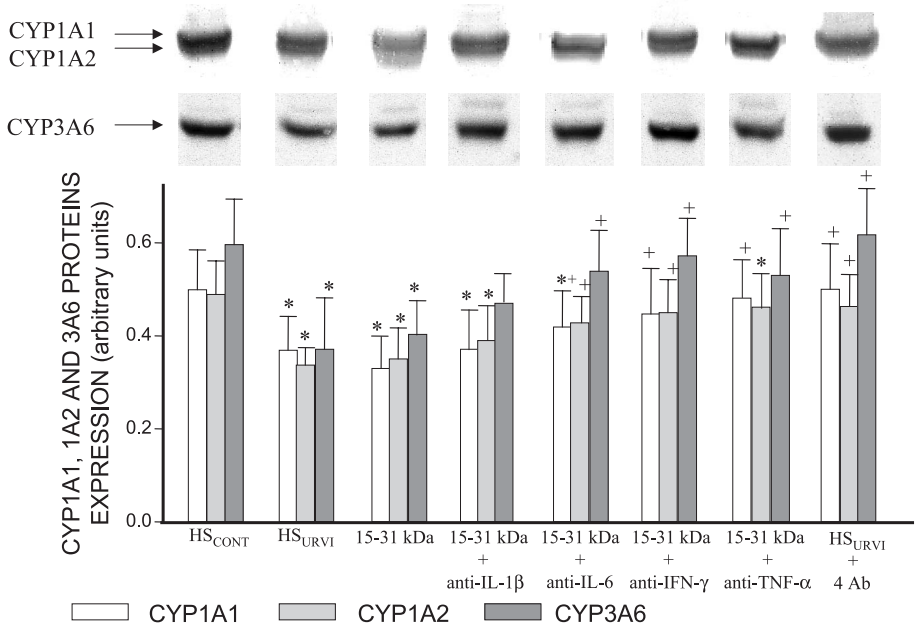

Fig. 3. Effect of anti-cytokine antibodies (Ab) on the ability of the 15–31-kDa HPLC fraction of serum from volunteers with an upper respiratory tract viral infection reaction (HS_{URVI}) to reduce the expression of CYP1A1, 1A2 and 3A6 proteins after 48 h of incubation with hepatocytes from control rabbits (H_{CONT}). The upper panel of the figure illustrates representative gels. HS_{CONT}: serum from healthy volunteers. Values on the abscissa correspond to Mr of the proteins contained in each fraction. Data are mean±S.E. of the ratio of the sample over the control. *P<0.05 compared with HS_{CONT} (n=4) or 15–31 kDa fraction (n=4), respectively.

ratory viral infection were screened for the presence of cytokines. Interleukin-6 was detectable in all serums, with a mean concentration of 8.6±1.6 pg/ml. Although interleukin-1β was not detectable, interferon-γ and tumour necrosis factor-α concentrations were above the detectable value of the assay in the serum of two individuals, with concentrations of 75 and 48 pg/ml, respectively.

After 48 h of incubation with hepatocytes, recombinant human cytokines (interleukin-1β, interleukin-6, interferon-γ and tumour necrosis factor-α) downregulated CYP1A1, 1A2 and 3A6 apoprotein, the effect being maximum at 10 ng/ml, with decreases ranging from 15% to 35%. The combination of the four cytokines (5 ng/ml each cytokine) produced a greater reduction than any individual cytokine incubated alone (n=2). A representative gel is shown in Fig. 4.

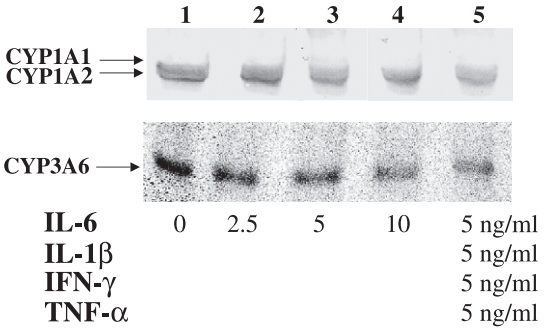

Fig. 4. Effect 0, 2.5, 5 and 10 ng/ml of recombinant human interleukin-6 (IL-6) (lanes 1–4) and of the combination of interleukin-6, interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (5 ng/ml) on the expression of CYP1A1, 1A2 and 3A6 proteins after a 48-h period of incubation with hepatocytes from control rabbits.

4. Discussion

This study demonstrates that sera from rabbits with an aseptic inflammatory reaction contain mediators capable to downregulate CYP1A1, 1A2 and 3A6 protein expression and to repress the content of mRNA encoding for these apoproteins. The mediators involved are interleukin-6 and interleukin-1β. On the other hand, sera from humans with an upper respiratory viral infection downregulate CYP1A1, 1A2 and 3A6 expression and the content of mRNA encoding for these proteins by means of interleukin-1β, interleukin-6, interferon-γ and tumour necrosis factor-α.

At the site of the injection of turpentine, the inflammatory response is initiated by the release of interleukin-1β and tumour necrosis factor-α from mast cells and macrophages, cytokines that stimulate the release of interleukin-6 from stromal and other cells (Watkins et al., 1995; Josephs et al., 2000; Labow et al., 1997). In response to a turpentine-induced inflammatory reaction, interleukin-6 and interleukin-1β are responsible for the increase in expression of acute phase proteins and the decrease in expression of albumin (Fantuzzi and Dinarello, 1998; Fantuzzi et al., 1997; Sillaber et al., 1993; Rosoff et al., 1998). Moreover, in the turpentine-induced inflammatory reaction, interleukin-6 reduces the content of mRNA encoding for *CYP1A2*, *CYP2A5* and *CYP3A11* proteins (Siewert et al., 2000; Jover

et al., 2002). The present results demonstrate that in vivo, in rabbits with a turpentine-induced inflammatory reaction, besides interleukin-6, interleukin-1β is a serum mediator that contributes to downregulate hepatic CYP1A1, 1A2 and 3A6 protein expression.

We have shown that incubation of serum from rabbits with a turpentine-induced inflammatory reaction with hepatocytes from rabbits with the inflammatory reaction for 4 h reduces the activity of CYP1A1, 1A2 and 3A6 without changing their expression, and interleukin-6 accounts for the decrease in activity (Bleau et al., 2000). The effect of serum from rabbits with a turpentine-induced inflammatory reaction on P450, e.g., decrease in activity or downregulation, appears modulated (a) by the incubation period, in the sense that short incubations decrease the activity and longer incubations reduce the expression and (b) by the serum mediators which depend upon the nature of the inflammatory reaction. The mechanism of action underlying the effects of serum from rabbits with a turpentine-induced inflammatory reaction on P450 also differ; the decrease in P450 activity appears associated to the presence of reactive oxygen intermediates, in particular nitric oxide and hydrogen peroxide. Effectively, inflammatory stimuli such as LPS and cytokines increases the expression of inducible nitric oxide synthase (NOS2), resulting in nitric oxide synthesis and inhibition of cytochrome *P*450 activity due to a direct effect on the enzymes (Stadler et al., 1994; Khatsenko et al., 1993; El-Kadi et al., 2000). On the other hand, the downregulation is exerted at the mRNA level.

The mediators in the serum from humans with an upper viral respiratory infection causing P450 downregulation show a certain degree of specificity concerning the isozymes affected. Interferon-γ and tumour necrosis factor-α mediate the downregulation of CYP1A1 and CYP1A2 proteins; in addition, interleukin-6 is involved in the suppression of CYP1A2. The decrease in expression of both apoproteins is probably associated to a reduction in the expression of their mRNA, e.g., secondary to a pre-translational mechanism. In the case of CYP3A6, the downregulation results from the action of interleukin-1β, interleukin-6, interferon-γ and tumour necrosis factor-α. The inability to detect interleukin-1β in the serum from subjects with an upper viral respiratory infection does not deter a role for interleukin-1β, since there is evidence that extremely small variations in interleukin-1β plasma concentrations, of the order of the femtomolar, may be enough to trigger an effect (Zheng et al., 1995).

Whole serum from subjects with an upper viral respiratory infection reduced CYP3A6 expression and *CYP3A6* mRNA, while the HPLC fractions diminished the apoprotein but not *CYP3A6* mRNA. This may be explained by the fact that 15–23- and 24–31-kDa fractions from two sera tended to increase *CYP3A6* mRNA, while the third sample decreased it. These results indicate that the mechanism of CYP3A6 regulation is complex. CYP3A6 expression can be depressed by pre-translational and translational mechanisms. It has been shown that interferon-γ reduces CYP3A expression without affecting *CYP3A* mRNA (Muntané-Relat et al., 1995) and that interferon-γ acts through a posttranscriptional mechanism mainly by inhibiting *CYP3A6* mRNA translation (Calleja et al., 1998). We ignore why the serum of two subjects with a viral infection elicited a pretranscriptional effect and the third triggered a translational effect. We may speculate that the response to the viral infection was rather variable among the volunteers, as evidenced by the fact that serum concentrations of interleukin-6, interferon-γ and tumour necrosis factor-α were inconstant. The increase in *CYP3A6* mRNA may be due to the presence of substances able to induce *CYP3A6* mRNA, e.g., cortisol, triggered by the infectious stress (Fantuzzi and Dinarello, 1996).

In hepatocytes, recombinant human interleukin-6 concentrations of the order of nanograms per milliliter were required to decrease the expression of CYP1A1, CYP1A2 and CYP3A6 proteins. In contrast, average concentration of interleukin-6 in the serum of subjects with an upper respiratory viral infection was 8.6±1.6 pg/ml. This 1000-fold concentration difference required to depress P450 expression cannot simply be explained on the basis of lower biological activity of the recombinant protein, but rather, it suggests that in vivo the cytokines trigger and additive and/or synergistic response. An additive and/or synergistic effect of several cytokines on P450 is supported by the fact that (a) the combination of the four recombinant human cytokines decreases the expression of CYP3A6 more than that produced by any single cytokine and (b) the neutralisation of all cytokines is required to revert totally the effect of serum from humans with an upper viral respiratory infection.

The present study emphasises how the nature of an inflammatory reaction influences the repressor effect of a given cytokine (Table 3). Several mechanisms must underlie these differences, such as the number of cytokines released, the concentrations of these cytokines, the cross-talk between cytokine signalling pathways and the modulation of a cytokine's effect by another cytokine (Ahmed and Ivashkiv, 2000). The effectiveness of an individual cytokine to downregulate selected P450 isoforms contrasts with the effect of

Table 3
Effect of several cytokines on the expression of CYP1A1, 1A2 and 3A6 depending upon the nature of the inflammatory reaction

		CYP1A1	CYP1A2	CYP3A6
IL-1β	RS_{TIIR}	−	+	+
	HS_{URVI}	−	−	+
IL-6	RS_{TIIR}	+	+	+
	HS_{URVI}	−	+	+
INF-γ	RS_{TIIR}	−	−	−
	HS_{URVI}	+	+	+
TNF-α	RS_{TIIR}	−	−	−
	HS_{URVI}	+	−	+

RS_{TIIR} is serum from rabbits with a turpentine-induced inflammatory reaction; HS_{URVI} is human serum from individuals with an upper respiratory viral infection. (−) denotes absence of effect, and (+) downregulation of the P450 isoform.

serum from rabbits with a turpentine-induced inflammatory reaction and serum from humans with a respiratory viral infection where several cytokines at rather low concentrations are present. For instance, in human hepatocytes, it has been reported that interleukin-1β is the strongest depressor of CYP1A2 and CYP3A4 expression, and that interferon-γ has a negligible effect on CYP3A4; moreover, interleukin-6 ability to depress CYP1A2 and 3A4 is similar to that of tumour necrosis factor-α (Abdel-Razzak et al., 1993). In rabbit hepatocytes, interleukin-1β does not affect CYP3A6, although interferon-γ decreases it by around 45% (Calleja et al., 1998). In mouse liver, interferon-α does not downregulate CYP1A1, although it reduces CYP1A2 expression (Stanley et al., 1991) and in the rat, polyinosinic acid–polycytidylic acid, a potent stimulator of interferon-α/β, does not modify the expression of constitutive CYP1A1 (Cribb et al., 1994).

In conclusion, the present results are direct evidence that in vivo, in an aseptic inflammatory reaction and in a viral infection of the upper respiratory tract, interleukin-1β, interleukin-6, interferon-γ and tumour necrosis factor-α are responsible for the downregulation of CYP1A1, 1A2 and 3A6. The reduction in expression of CYP1A1 and 1A2 appears secondary to a pre-translational mechanism, and the decrease in CYP3A6 expression could be due to pre- and translational mechanisms. Moreover, this study demonstrates that the serum mediators responsible for the downregulation of hepatic P450 (a) differ from the mediators in serum responsible for P450 decrease in activity, where the effect of interleukin-6 and interferon-γ appears prominent, (b) differ depending upon the nature of the inflammatory reaction and (c) elicit an effect that differs from that elicited by individual cytokines.

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