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Hypoxia-induced down-regulation of CYP1A1/1A2 and up-regulation of CYP3A6 involves serum mediators

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1 Acute moderate hypoxia modifies the catalytic activity and expression of certain isoenzymes of hepatic cytochrome P450 (P450). The aim of this study was to document whether hypoxia affects hepatic P450 directly or through the release of serum mediators.

2 Rabbits were subjected to a FiO_2 of 8% for 48 h, sacrificed, and serum and hepatocytes were isolated; hepatocytes from control and rabbits with hypoxia were incubated with serum from control and hypoxic rabbits for 4 and 24 h, and total P450 content, CYP1A1, 1A2 and 3A6 activities and expressions were assessed. Sera were fractionated by size exclusion chromatography and fractions tested for their ability to modify activity and amount of P450, and serum mediators were identified through neutralization experiments.

3 Total serum and fractions with proteins of 15–23 and 65–94 kDa of M_r reduced P450 content and expression of CYP1A1, 1A2 and 3A6, as well as *CYP1A1*, *1A2* and *3A6* mRNA. Total serum and the fraction with 32–44 kDa proteins increased CYP3A6 activity and protein and mRNA. The serum mediators implicated in the decrease in activity and expression of CYP1A1, 1A2 and 3A6 were interferon-γ (IFN-γ), interleukin-1β (IL-1β) and IL-2. Erythropoietin (Epo) was partly responsible for the increase in P450 content and CYP3A6 expression.

4 In conclusion, acute moderate hypoxia diminishes the activity and expression of CYP1A1, 1A2 and *CYP1A1*, *1A2* mRNA, and increases CYP3A6 protein, activity and *CYP3A6* mRNA. Several mechanisms contribute to these changes in P450, among them the release of cytokines acting as serum mediators.

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Abbreviations: AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; 1,3DMU, 1,3-dimethyluric acid; DFB (3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulphonylphenyl)-(5H)-furan-2-one; DFH 3-hydroxy-5,5-dimethyl-4-(4-methylsulphonylphenyl)-(5H)-furan-2-one; Epo, erythropoietin; FiO_2, fractional concentration of inspired O_2; H_{CONT}, hepatocytes from control rabbits; 13-HETE, 13-hydroxyeicosatrienoic acid; H_{HYPO}, hepatocytes from rabbits with hypoxia; HIF-1, hypoxia-inducible factor 1 transcriptional activator; H_2O_2, hydrogen peroxide; IFN-γ, interferon-γ; IL-1β, interleukin-1β; M_r, relative molecular mass; 1MU, 1-methyluric acid; 3MX, 3-methylxanthine; NO•, nitric oxide; P450, cytochrome P450; PI3-K, phosphatidylinositol 3-kinase; ROI, reactive oxygen intermediates; S_{CONT}, serum from control rabbits; S_{HYPO}, serum from rabbits with hypoxia; TNF-α, tumour necrosis factor; WME, William's medium E

Introduction

The clearance of theophylline can be decreased in patients with chronic obstructive lung disease, pulmonary oedema, pulmonary heart disease or congestive heart failure (Powell *et al*., 1978). Since patients with these disease states present episodes of acute hypoxia, the decrease in theophylline metabolic clearance has been ascribed to hypoxia (Richer & Lam, 1993). Early *in vivo* and *in vitro* studies supported that acute hypoxia reduces the activity of multiple biotransformation pathways (Jones, 1981). Modulation of cytochrome P450 (P450) by hypoxia was confirmed *in vivo* in animals with acute moderate hypoxia where the clearance of theophylline was reduced (Letarte & du Souich, 1984). Moreover, subchronic hypoxia also reduces the activity and expression of enzymes involved in the biotransformation of drugs (Shan *et al*., 1992). Supporting that in man hypoxia modulates P450, it has been shown that in patients with congestive heart failure, total hepatic P450 content is reduced, as well as the expression of CYP1A1 and 1A2 isoenzymes (Ng *et al*., 2000). Interestingly, *in vivo*, in rabbits subjected to acute hypoxia, the expression of CYP3A6 is enhanced (Kurdi *et al*., 1999).

In vivo, the effect of hypoxia on P450 resembles that elicited by an acute local inflammatory reaction, e.g. there is a decrease in activity and down-regulation of several P450 isoforms, with the exception that an inflammatory reaction also down-regulates CYP3A (Morgan, 1997). In rabbits with a turpentine-induced inflammatory reaction, interleukin-6

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(IL-6) is the serum mediator responsible for the decrease in activity of P450 isoforms; IL-1β, tumour necrosis factor (TNF-α) and interferon-γ (IFN-γ) have a minor role (Bleau *et al*., 2000).

Hypoxia prompts the release of numerous cytokines, such as IL-1β, IL-2, IL-4, IL-5, IL-6, TNF-α and IFN-γ (Naldini *et al*., 1997), and erythropoietin (Epo) (Lacombe & Mayeux, 1999). We speculated that these cytokines might mediate the changes in hepatic P450 isoforms in animals subjected to acute moderate hypoxia. This hypothesis was supported by the fact that these cytokines can induce changes in the expression of P450 genes (Calleja *et al*., 1997; 1998).

The aims of the present study were to assess whether acute moderate hypoxia triggers the release of serum mediators eventually leading to changes in P450 content and activity. To document the presence of serum mediators, P450 content, activity and amount of CYP1A1, 1A2 and 3A6 apoproteins and genes were assessed following 4 and 24 h incubation periods of hepatocytes with sera from rabbits with acute moderate hypoxia. Mediators in sera were isolated by size-exclusion high performance liquid chromatography, and the potential effect of IL-2, IL-1β, IL-6, IFN-γ and Epo, on P450 isoforms was counterbalanced by direct neutralization with antibodies. In rabbits with moderate acute hypoxia, IFN-γ is the primary serum mediator responsible for the depression of CYP1A1 and 1A2 proteins and mRNA, and Epo is partly responsible for the up-regulation of CYP3A6 protein and gene.

Methods

Animals and hepatocyte collection

Male New Zealand White rabbits (1.8–2.2 Kg) were obtained from Ferme Charles Rivers (St-Constant, Quebec, Canada). Rabbits were maintained on Purina Laboratory Chow and water *ad libitum* for at least 7 days before any experimental work was undertaken. To induce the hypoxia, rabbits were introduced in a plexiglas chamber ($0.75 \times 1.20 \times 1.25$ m^3) with a fractional concentration of inspired O_2 (FiO_2) of 8%, adjusted with an oxygen monitor (OM-15, Sensor Medics Corp., CA, U.S.A.) connected to an electrovalve (Asco Valves, Brantford, Ontario, Canada) that allowed the access of nitrogen into the chamber which displaced the air off. All the rabbits had access to Purina Laboratory Chow and water *ad libitum* for the 48 h that lasted the hypoxia. Control rabbits were also placed into the chamber for the experiments, but breathing room air ($FiO_2 = 21\%$). All the experiments were conducted according to the Canadian Council on Animal Care guidelines for use of laboratory animals.

Hepatocytes from rabbits with hypoxia (H_{HYPO}) and from control rabbits (H_{CONT}) were isolated 48 h after the induction of hypoxia or breathing room air, respectively, according to the two-step liver perfusion method of Seglen (1976) with minor modifications (El-Kadi *et al*., 1997). Briefly, rabbits were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}), a midline laparotomy was performed and the portal, the suprahepatic and inferior cava veins were cannulated. The liver was perfused *via* the portal vein with a solution containing (mM) NaCl 15, KCl 5, KH_2PO_4 1, HEPES 25, EGTA 0.5, glucose 5.5, and 56.8 mg ml^{-1} heparin with a peristaltic pump (Harvard Apparatus Co., Inc., U.S.A.), followed by a perfusion with a solution of 0.013% collagenase, 1 mM $CaCl_2$ and 0.25 mM trypsin inhibitor. All solutions were maintained at 37°C and saturated with 100% O_2. The liver was maintained wet with saline during the entire period of perfusion. Living cells were isolated on a 40% Percoll gradient. Viability was assessed by Trypan blue exclusion to ensure that it was greater than 90%; viability was not affected by hypoxia or any experimental condition. Cell concentration was adjusted to 4×10^6 ml^{-1} with William's medium E (WME) supplemented with 10% calf serum and 1 μM insulin. Aliquots of 2 ml of the hepatocytes in suspension were transferred into 6-well plastic culture plates (Falcon, Becton Dickinson Labware, Rutherford, NJ, U.S.A.) coated with type 1 rat tail collagen and incubated for 4 and 24 h at 37°C in an atmosphere of 95% O_2 and 5% CO_2. Cell cultures were always conducted under sterile conditions.

Rabbit serum preparation

Blood samples (10 ml) were withdrawn from the rabbits 48 h after the induction of hypoxia and from control rabbits in a sterile Vacutainer Brand SST (Becton Dickinson, Mississauga, ON, Canada). Blood samples were allowed to clot at room temperature for 2 h, thereafter were centrifuged at 2500 r.p.m. for 5 min, and the serum was decanted and stored frozen at −20°C in 1 ml aliquots until use. Preliminary studies have shown that when samples were handled as described, serum mediators conserved their activity for up to 12 months.

Fractionation of serum proteins

Serum proteins were separated by size exclusion high performance liquid chromatography (HPLC) on a Superose 12 HR column from Pharmacia Biotech (Baie d'Urfé, Quebec, Canada). Flow rate of the mobile phase was set at 0.3 ml min^{-1} and column pressure was maintained between 9–12 bar with a LKB 2150 HPLC pump (Bromma, Sweden). Absorbance was measured at 280 nm with a Waters 490E spectrophotometric detector (Millipore, Milford, MA, U.S.A.). The eluant buffer included (mM) NaCl 115, KCL 5, KH_2PO_4 1, HEPES 1, EGTA 25, and glucose 5.5; the pH of the solution was adjusted to 7.4 and filtered through a nylon mesh (pore size 0.22 μm). Serum aliquots of 300 μl were injected into the column and fractions of 1.2 ml were collected with a fraction collector (LKB 2211 Superrac). To calculate the relative molecular mass (M_r) of the serum proteins contained in each HPLC fraction, a calibration curve was established by injecting 300 μl of the buffer containing a mixture of six standard proteins (100 μg ml^{-1}): 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). The proteins isolated in the first fraction were assumed to have a M_r greater than 95 kDa, considering that the column sorts out proteins with a M_r lower than 95 kDa.

In order to increase inhibitory activity of the fractions collected, these were concentrated on Microsep 3K membranes (Pall Filtron, Northborough, MA, U.S.A.) which

retain proteins greater than 3 kDa. Three millilitres of the fractions were added to the sample reservoir and centrifuged at $75{,}000 \times g$ to reduce the volume and hence concentrate serum fractions 1.25 times.

Experimental protocol

The efficiency of serum mediator(s) to modify hepatic P450 content and activity was characterized by incubating hepatocytes from control rabbits and from rabbits with hypoxia with 200 μl of total serum or 200 μl of the HPLC fractions for 4 and 24 h, and assessing total P450 content and activity. Controls included hepatocytes incubated for 4 and 24 h in absence of serum, and H_{CONT} incubated for 24 h with HPLC fractions of serum from control rabbits (S_{CONT}). Hepatic P450 content was measured spectrophotometrically in cell lysates as described by Omura & Sato (1964). The amount of proteins in hepatocytes was measured in cell lysates by the method of Lowry *et al*. (1951).

The effect of serum mediators on the activity of CYP1A1 and 1A2 was determined by measuring the ability of the hepatocytes to biotransform theophylline to 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU) (Kurdi *et al*., 1999). Theophylline was dissolved in serum-free WME, and 100 μl were added to each well and incubated for 4 and 24 h with the hepatocytes at a final concentration of 176 μM. At time zero, 350 μl of the supernatant were collected from each well (control sample). The remaining supernatant was collected following 4 and 24 h of incubation and frozen at -20°C until theophylline, 3MX, 1MU and 1,3DMU were assayed by HPLC (Kurdi *et al*., 1999). The effect of serum mediators on the activity of CYP3A6 was determined by measuring the ability of the hepatocytes to convert 3,4-difluorobenzyloxy)-5,5-dimethyl-4-(4-methylsulphonylphenyl)-(5H)-furan-2-one (DFB), a reported CYP3A4 probe in humans and rabbits to DFH, its fluorescent des-(difluoro)-benzyl metabolite (Chauret *et al*., 1999; unpublished observations). Incubations were performed according to a published procedure (Silva & Nicoll-Griffith, 2001). Briefly, 60 μM DFB was incubated with the hepatocytes for 15 min. An aliquot of the media was then transferred to a microtiter plate and quenched with an equal volume of acetonitrile containing 40% TRIS buffer (0.05 M). The fluorescence of the metabolite DFH was measured at excitation and emission wavelengths of 360 nm and 440 nm, respectively, using a fluorescent plate reader Wallac Victor^2 1420 Multilabel Counter, and expressed in arbitrary units.

The information about the activity and expression of P450 isoenzymes in hepatocytes obtained after 4 h of incubation with saline, e.g. in absence of serum or its fractions, was assumed to reflect the repercussions of 48 h of *in vivo* hypoxia on hepatic P450.

Western blot analysis

Hepatocytes were washed, harvested in ice-cold PBS and centrifuged at $1500 \times g$ for 5 min. The pellet was resuspended in cold lysis buffer (mM) HEPES 10 pH 7.9, KCl 10, EDTA 0.1, EGTA 0.1, dithiothreitol 1, protease inhibitor mixture, and cells were allowed to swell on ice for 15 min, and vortexed for 30 s. For Western blot analysis, 50 μg of cell lysate were separated by SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide) (Smith, 1994). Separated proteins were electrophoretically transferred to a nitrocellulose membrane using a semi-dry transfer process (Bio-Rad, Hercules, CA, U.S.A.). CYP1A1 and 1A2 were detected with a polyclonal anti-rabbit CYP1A1 (Oxford Biochemical Research, Oxford, MI, U.S.A.) diluted 1 : 100 in 5% nonfat milk in PBS/0.1% Tween 20 and visualized with an alkaline phosphatase conjugated secondary antibody using nitro blue tetrazolium as substrate (Kruger, 1994). CYP3A6 was detected with a monoclonal anti-rat CYP3A1 (Oxford Biochemical Research, Oxford, MI, U.S.A.) diluted 1 : 500 in 5% nonfat milk in PBS/0.1% Tween 20 using a secondary antibody conjugated with chemiluminescence reagent (horseradish peroxidase enzyme) and visualized by autoradiography (Thorpe *et al*., 1985). The assay was linear in the range of protein amounts assessed under the present experimental conditions. The intensities of the bands were measured with the software Un-Scan-It-Gel (Silk Scientific Inc., Orem, UT, U.S.A.) and are represented in arbitrary units.

Northern blot analysis of CYP1A1*,* 1A2 *and* 3A6 *mRNAs*

Following 4 or 24 h of incubation, liver cells were washed in 3 ml PBS and flash-frozen in liquid nitrogen. Samples were kept at -80°C until RNA extraction and quantification according to the method described by Leblond *et al*. (2001). Total RNA was isolated using 1 ml of TRIZOL Reagent (Life Technologies Inc.) per $5-10 \times 10^6$ cells. RNA concentration was measured spectrophotometrically at the absorbance of 260 nm ($A_{260/280}$ ratio ≈ 2). Total RNA samples were denatured by heating at 60°C for 10 min in buffer containing 30 mM 4-morpholinopropanesulphonic acid, 42% deionized formamide, and 8.5% formaldehyde. Thirty micrograms of RNA species were then separated by electrophoresis through a denaturing 1% agarose–1.7% formaldehyde gel submerged in 20 mM 4-morpholinopropanesulphonic acid, 8 mM sodium acetate and 1 mM EDTA buffer, 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 prehybridized for 2 h at 52°C with 250 μg ml^{-1} denatured salmon sperm DNA, 1% dextran sulphate, 1% bovine serum albumin, 1 mM EDTA, 7% SDS, and 0.5 M NaPO4 (pH 7.2). The cDNA probes (rabbit CYP1A1, 1A2, 3A6 and rat 18S) were labelled with [α-^{32}P]dCTP (3000 Ci mmol^{-1}; Amersham Pharmacia Biotech) using Klenow fragment according to the oligo-priming method of the Oligolabelling kit (Amersham Pharmacia Biotech). Hybridization was performed at 52°C for 24 h with the radiolabelled cDNA probe in the prehybridization buffer. Blots were washed at 65°C for 10 min with a solution containing 3 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 0.02 M EDTA, pH 7.4 and 0.1% SDS. Membranes were exposed to an autoradiography film Biomax by means of Biomax Tran-Screen-HE intensifying screens (Kodak) at -80°C during 24 h. The intensities of the bands were measured with the software Un-Scan-It-Gel and are represented in arbitrary units. The assay is linear in the range of protein amounts assessed under the present experimental conditions.

There are two forms of CYP3A6 mRNA expressed in the liver, one with 1.85-kb and the other with 1.7-kb. Even if

these forms differ by the length of their 3′ untranslated region, they originate the same protein (Dalet *et al*., 1988). Both forms can be separated and quantified simultaneously.

Immuno-neutralisation of cytokines

The selection of the antibodies used for the immuno-neutralization of cytokines was based (a) upon the M_r of the proteins incorporated in the HPLC fractions having the ability to change P450 content and activity, and (b) according to the kind of cytokines hypoxia releases (Naldini *et al*., 1997). To prevent the changes in P450 content and activity induced by the HPLC fractions, the following antibodies were used: a goat anti-rabbit IL-1β (anti-IL-1β) polyclonal antibody, and an anti-human IL-2 (anti-IL2), an anti-human IFN-γ (anti-IFN-γ), an anti-human IL-6 (anti-IL-6), and an anti-human Epo (anti-Epo) monoclonal antibodies. The antibodies against human proteins were used to neutralize the homologous rabbit proteins because of the known inter-species reactivity of these antibodies (Huang *et al*., 1997; Muscettola *et al*., 1995). An irrelevant monoclonal antibody (IgG to *Pseudomona aeruginosa*) served as control. Aliquots of 2 μg of each antibody were added individually to 200 μl of the HPLC fractions having the ability to change P450 content and activity, and were incubated at 37°C for 1 h. The antibody and the HPLC fractions were added to the hepatocytes at the beginning of the 4 and 24 h periods of incubations and activity and content of P450 was assessed. The amount of antibodies used, e.g. 2 μg, was selected because it was proven that 2 μg were effective to immuno-neutralize IL-1β, IL-6, and IFN-γ in the sera of rabbits and of humans with an inflammatory reaction (Bleau *et al*., 2000). In the present study, 2 and 4 μg of anti-IL-6 antibody were used.

Drugs and chemicals

Percoll gradient, William's medium E, calf serum, type I rat tail collagen, NaCl, KCl, KH_2PO_4, HEPES, EGTA, glucose, theophylline, 3MX, 1MU and 1,3DMU were purchased from Sigma Chemicals (Sigma, St. Louis, MO, U.S.A.). DFB and DFH were provided by Merck Frosst Canada (Kirkland, Québec, Canada). Insulin was acquired from Boehringer Mannheim Biochemica (Mannheim, Germany), and L-glutamic dehydrogenase, aldolase triosephosphate isomerase, trypsin inhibitor, cytochrome *c*, and aprotinin from Pharmacia Biotech (Baie d'Urfé, QC, Canada). The polyclonal anti-rabbit CYP1A1 and the monoclonal anti-rat CYP3A1 antibodies were purchased from Oxford Biochemical Research (Oxford, MI, U.S.A.), the anti-IL-1β antibody from Cedar Lane (Hornby, ON, Canada), the anti-IL-2, anti-IL-6, and anti-IFN-γ antibodies from R&D Systems (Minneapolis, MN, U.S.A.). Finally, anti-Epo antibody was purchased from Genzyme Diagnostics (Cambridge, MA, U.S.A.). Specific cDNA probes for rabbit CYP1A1, 1A2 and 3A6 were kindly provided by Prof P. Maurel (INSERM U128, Montpelier, France) and rat 18S by Dr V. Pichette (Hôpital Maisonneuve-Rosemont, Montréal, Canada).

Statistical analysis

All results are presented as mean$\pm$s.e.mean. The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one-way analysis of variance (ANOVA), followed by the Newman-Keuls *post hoc* test. Differences were considered significant when $P<0.05$.

Results

Effect of hypoxia in vivo *on hepatic P450 content, activity and amount of P450 isoforms*

Exposure of rabbits ($n=7$) to an 8% FiO_2 generated a stable hypoxemia, with an average PaO_2 of 34.2 ± 1.3 mmHg, without influencing the $PaCO_2$ ($\approx20.7\pm1.0$) and arterial pH (7.47 ± 0.05). Compared with H_{CONT}, 48 h of hypoxia *in vivo* reduced total P450 content by 45% and decreased 3MX, 1MU and 1,3DMU output by 58, 42 and 33% ($P<0.05$), respectively (Table 1).

Compared with rabbits ($n=7$) breathing a 21% FiO_2, e.g. room air, hypoxia reduced the amount of CYP1A and 1A2 proteins by 37 and 40%, respectively (Figure 1A). In parallel, *CYP1A* and *1A2* mRNAs were decreased in animals exposed to 8% FiO_2 (Figure 2A). Forty-eight hours of hypoxia increased the amount of CYP3A6 by 70% (Figure 1A) as well as the two forms of *CYP3A6* mRNA (Figure 2A). This increase in CYP3A6 expression resulted in the enhancement of CYP3A6 activity, that is the formation of the fluorescent metabolite DFH was $\approx$76% higher ($P<0.05$) in H_{HYPO} than in H_{CONT}, e.g. 11627 ± 288 and 6606 ± 245.

Effect of S_{CONT} and serum of rabbits with hypoxia (S_{HYPO}) on H_{CONT} following 4 and 24 h of incubation

Incubation for 4 h of H_{CONT} with S_{CONT} ($n=7$) and S_{HYPO} ($n=7$) did not modify P450 content, theophylline biotransformation (Table 1) or the amount of CYP1A1, 1A2 and 3A6 proteins (data not shown).

Compared with H_{CONT} incubated with S_{CONT}, 24 h of incubation of H_{CONT} with S_{HYPO} ($n=7$), reduced the output of 3MX, 1MU and 1,3DMU by 26, 31 and 21% ($P<0.05$), respectively, increased the amount of CYP3A6 by 28% (data not shown) and the formation of DFH by 29% ($P<0.05$), e.g. 4842 ± 191 for S_{CONT} and 6246 ± 202 for S_{HYPO}. No changes in P450 content (Table 1) or amount of CYP1A1, 1A2 proteins (data not shown) were observed. However, S_{HYPO} diminished ($P<0.05$) the amount of *CYP1A1* mRNA by 20%, e.g. 0.531 ± 0.027 with S_{CONT} and 0.425 ± 0.014 with S_{HYPO}, and that of *CYP1A2* mRNA by 24%, e.g. 0.424 ± 0.041 with S_{CONT} and 0.322 ± 0.014 with S_{HYPO}. On the other hand, S_{HYPO} increased the amount of *CYP3A6* mRNA by 49%, e.g. 0.120 ± 0.020 with S_{CONT} and 0.179 ± 0.006 with S_{HYPO} ($P<0.05$, $n=4$; Figure 2B).

Effect of S_{CONT} and S_{HYPO} on H_{HYPO} following 4 and 24 h of incubation

Compared with S_{CONT} ($n=18$), 4 h of incubation of S_{HYPO} ($n=18$) with H_{HYPO} did not modify P450 content or amount of CYP1A1, 1A2 and 3A6 proteins. However, S_{HYPO} reduced the output of 3MX, 1MU and 1,3DMU by 31, 29 and 37%, respectively ($P<0.05$) (Table 2).

Table 1 Effect of 48 h hypoxia *in vivo* and of the incubation of control hepatocytes with serum of control and hypoxic rabbits for 4 and 24 h on P450 content and metabolism theophylline

	Cytochrome P450 (nmol mg^{-1} protein)	*3MX* ($\mu g\ ml^{-1}$)	*1MU* ($\mu g\ ml^{-1}$)	*1,3DMU* ($\mu g\ ml^{-1}$)
In vivo hypoxia				
H_{CONT} + NaCl	0.330 ± 0.021	0.094 ± 0.011	0.140 ± 0.006	1.340 ± 0.116
H_{HYPO} + NaCl	0.181 ± 0.020*	0.039 ± 0.004*	0.081 ± 0.003*	0.901 ± 0.043*
Incubation 4 h				
H_{CONT} + S_{CONT}	0.361 ± 0.022	0.099 ± 0.010	0.120 ± 0.004	1.335 ± 0.201
H_{CONT} + S_{HYPO}	0.349 ± 0.020	0.091 ± 0.021	0.111 ± 0.013	1.195 ± 0.111
Incubation 24 h				
H_{CONT} + S_{CONT}	0.325 ± 0.031	0.222 ± 0.031	0.249 ± 0.019	4.268 ± 0.321
H_{CONT} + S_{HYPO}	0.330 ± 0.024	0.164 ± 0.022*	0.171 ± 0.015*	3.341 ± 0.303*

Data is presented as mean ± s.e.mean. Hepatocytes from control (H_{CONT}) and rabbits with acute moderate hypoxia (H_{HYPO}) were incubated with NaCl 0.9% ($n=7$) or with serum from control rabbits (S_{CONT}) ($n=7$) and serum from rabbits with acute moderate hypoxia (S_{HYPO}) ($n=7$) for 4 and 24 h. 3MX, 1MU and 1,3DMU are 3-methylxanthine, 1-methyluric acid and 1,3-dimethyluric acid, respectively. *$P<0.05$ compared with control.

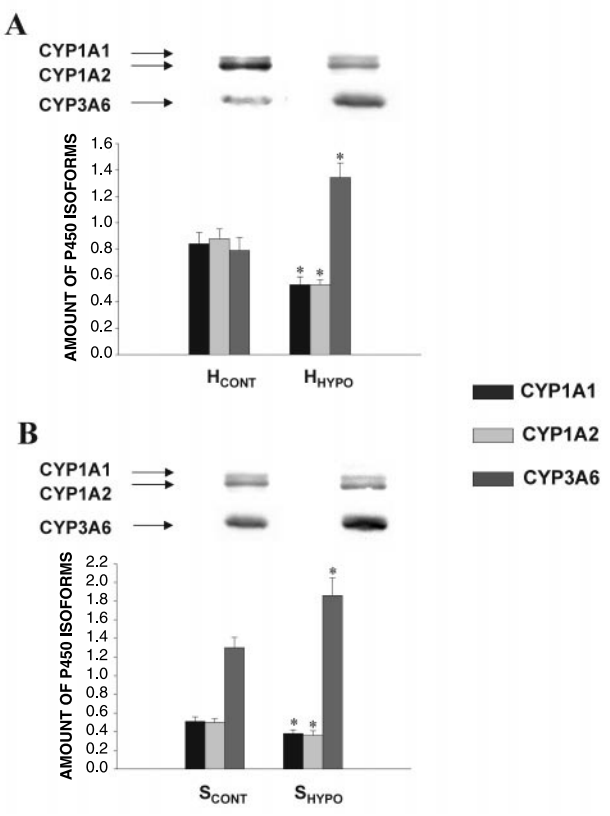

Figure 1 Effect (A) of 48 h acute moderate hypoxia *in vivo* on the amount of CYP1A1, 1A2 and 3A6 apoproteins in hepatocytes ($n=7$), and (B) of serum from rabbits with acute moderate hypoxia (S_{HYPO}) ($n=7$) on the amount of CYP1A1, 1A2 and 3A6 apoproteins incubated for 24 h with hepatocytes from rabbits with acute moderate hypoxia (H_{HYPO}). Bands are representative Western blots of CYP1A1, 1A2 and 3A6. Data is presented in arbitrary units as mean ± s.e.mean. *$P<0.05$ compared with control.

Following 24 h incubation, S_{HYPO} ($n=18$) did not affect P450 content in H_{HYPO}, but reduced the output of 3MX, 1MU and 1,3DMU ($P<0.05$) (Table 2). In addition, S_{HYPO} reduced the amount of CYP1A1 and 1A2 proteins by an average of 25 and 28%, respectively ($P<0.05$), and increased the expression of CYP3A6 by an average of 43% ($P<0.05$) (Figure 1B). However, S_{HYPO} did not diminish the amounts of *CYP1A1* and *1A2* mRNA, but increased the amount of *CYP3A6* mRNA by 59%, e.g. 0.358 ± 0.045 with S_{CONT} and 0.570 ± 0.016 with S_{HYPO} ($P<0.05$, $n=4$; Figure 2C). The activity of CYP3A6 was increased by 30% ($P<0.05$) e.g. 8702 ± 299 with S_{CONT} and 11312 ± 268 with S_{HYPO}.

Effect of HPLC fractions of S_{HYPO} on P450 content, activity and isoforms of H_{HYPO} following 4 and 24 h of incubation

Serum fractions of S_{CONT} when incubated for 4 h with H_{CONT} did not modify the amount and activity of total P450 (data not shown). Incubation of the HPLC fractions of S_{HYPO} ($n=12$) with H_{HYPO} for 4 h did not modify P450 content. However, the amount of theophylline metabolites generated by H_{HYPO} was decreased by the fractions containing proteins with a M_r of 15–23 kDa and of 65–94 kDa (Figure 3), despite that these two fractions did not affect the amount of CYP1A1, 1A2 and 3A6 proteins.

The serum fractions of S_{CONT} incubated for 24 h with H_{CONT} did not modify the expression and activity of CYP1A1, 1A2 and 3A6 (data not shown). Incubation of the HPLC fractions of S_{HYPO} ($n=12$) with H_{HYPO} for 24 h shows that the 15–23 kDa fraction decreased P450 content by 38% ($P<0.05$), while the 32–44 kDa fraction increased P450 content by 33% ($P<0.05$) (Figure 4). The 15–23 kDa and 65–94 kDa HPLC fractions reduced the output of 3MX, 1MU and 1,3DMU ($P<0.05$) (Figure 5). In addition, the 15–23 kDa fraction ($n=7$) reduced the amount of CYP1A1 and 1A2 proteins by 42 and 30%, respectively ($P<0.05$), and the amount of CYP3A6 by 21% (Figure 6). On the other hand, the amount of CYP3A6 protein was increased by 51% ($P<0.05$) by the 32–44 kDa fraction ($n=7$) (Figure 6).

Identification of the mediators in S_{HYPO} responsible for the changes in the amount and activity of P450

Compared with the biotransformation of theophylline by H_{HYPO} in presence of S_{CONT}, incubation of H_{HYPO} for 4 h with the 15–23 kDa and 65–94 kDa fractions of S_{HYPO} in

presence of anti-IFN-γ antibody ($n=6$), completely abrogated the decrease of theophylline metabolism elicited by these fractions (Figure 7). On the other hand, anti-IL-1β and anti-IL-2 antibodies ($n=6$) only partially reverted the decrease in theophylline metabolism elicited by the 15–23 kDa and 65–94 kDa fractions. The presence of anti-IL-6 antibody ($n=6$) did not prevent the effect of these two HPLC fractions on the output of 3MX, 1MU and 1,3DMU (Figure 7).

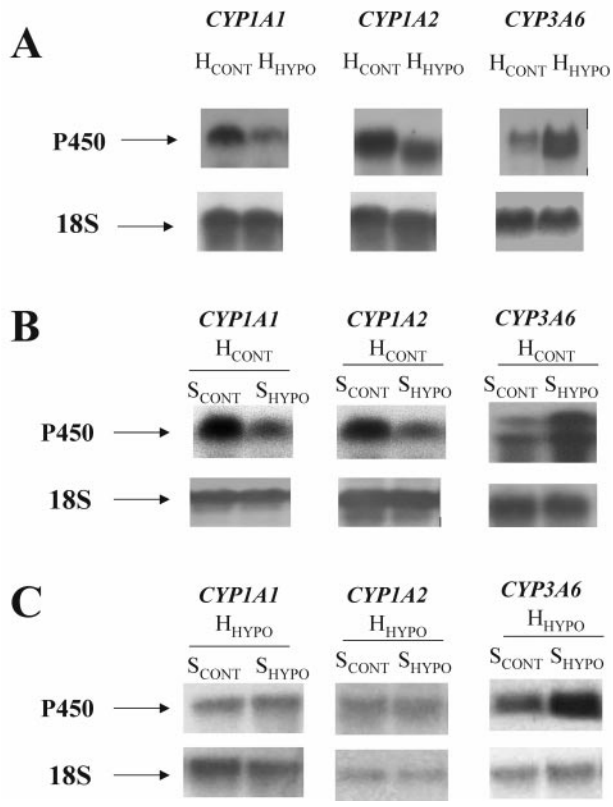

Figure 2 Effect (A) of *in vivo* 48 h acute moderate hypoxia on the amount of *CYP1A1*, *1A2* and *3A6* mRNA in hepatocytes of control rabbits (H_{CONT}) and rabbits subjected to hypoxia (H_{HYPO}); (B) effect of serum from control rabbits (S_{CONT}) and rabbits with acute moderate hypoxia (S_{HYPO}) on the amount of *CYP1A1*, *1A2* and *3A6* mRNA incubated for 24 h with hepatocytes from control rabbits (H_{CONT}); and (C) effect of S_{CONT} and S_{HYPO} on the amount of *CYP1A1*, *1A2* and *3A6* mRNA following 24 h incubation with hepatocytes from rabbits with hypoxia (H_{HYPO}).

Following 24 h incubation of H_{HYPO} with the 15–23 kDa fraction of S_{HYPO}, P450 content was reduced by 38% ($n=12$), and the addition of anti-IL-2, anti-IL-1β and anti-IFN-γ antibodies ($n=6$) to the 15–23 kDa fraction incremented P450 content to 84, 80 and 92% of control values, respectively ($P<0.05$) (Figure 8). Anti-IFN-γ, -IL-1β, -IL-2 and -IL-6 antibodies added to the 15–23 kDa and 65–94 kDa fractions elicited a variable effect on the output of 3MX, 1MU and 1,3DMU (Figure 9). Anti-IFN-γ antibody ($n=6$) prevented the effect of the two HPLC fractions, whereas anti-IL-2 ($n=6$) and anti-IL-1β antibodies ($n=6$) only partially, and anti-IL-6 antibody ($n=6$) did not elicit any effect, despite that the amount of antibody was increased to 4 μg. The addition of anti-IL-2, anti-IL-1β and anti-IFN-γ antibodies ($n=6$) to the 15–23 kDa fraction augmented the amount of CYP1A1 protein to 84, 80 and 90% of control values; only anti-IFN-γ antibody ($n=6$) increased CYP1A2 protein to 89% of control values (Figure 10).

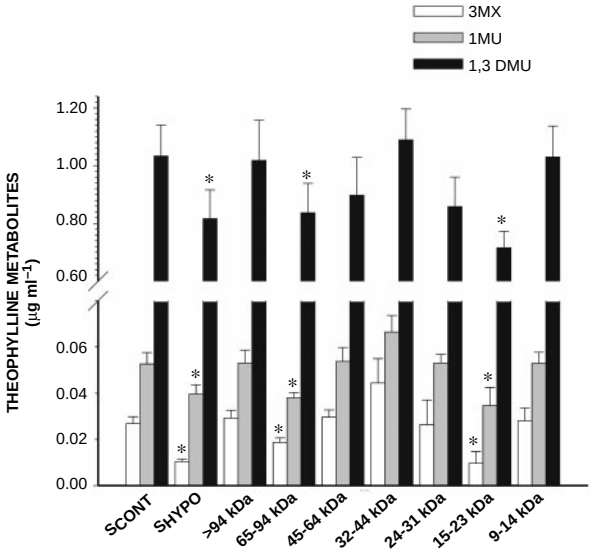

Figure 3 Effect of serum of control rabbits (S_{CONT}) ($n=10$), serum from rabbits with acute moderate hypoxia (S_{HYPO}) ($n=12$), and HPLC fractions of S_{HYPO} ($n=12$) incubated for 4 h with hepatocytes from rabbits with acute moderate hypoxia on the formation of theophylline metabolites. Data is presented as mean±s.e.mean. *$P<0.05$ compared with S_{CONT}.

Table 2 Effect of incubation of hepatocytes from hypoxic rabbits with serum from control and hypoxic rabbits for 4 and 24 h on P450 content and metabolism of theophylline

	Cytochrome P450 (nmol mg^{-1} protein)	*3MX* (μg ml^{-1})	*1MU* (μg ml^{-1})	*1,3DMU* (μg ml^{-1})
4 h incubation				
$H_{HYPO}+S_{CONT}$	0.16±0.019	0.035±0.003	0.085±0.002	1.112±0.036
$H_{HYPO}+S_{HYPO}$	0.15±0.010	0.024±0.001*	0.060±0.004*	0.701±0.015*
24 h incubation				
$H_{HYPO}+S_{CONT}$	0.12±0.010	0.078±0.005	0.090±0.011	2.106±0.165
$H_{HYPO}+S_{HYPO}$	0.11±0.010	0.059±0.003*	0.057±0.007*	1.295±0.120*

Data is presented as mean±s.e.mean. Hepatocytes from rabbits with acute moderate hypoxia (H_{HYPO}) were incubated with serum from control rabbits (S_{CONT}) ($n=18$) and serum from rabbits with acute moderate hypoxia (S_{HYPO}) ($n=18$) for 4 and 24 h. 3MX, 1MU and 1,3DMU are 3-methylxanthine, 1-methyluric acid and 1,3-dimethyluric acid, respectively. *$P<0.05$ compared with $H_{HYPO}+S_{CONT}$.

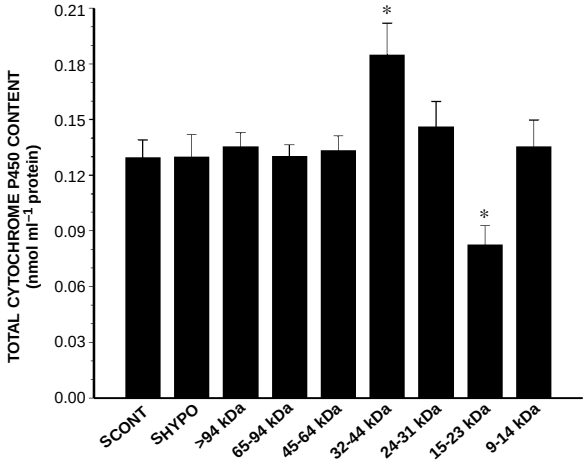

Figure 4 Effect of serum of control rabbits (S_{CONT}) ($n=10$), serum from rabbits with acute moderate hypoxia (S_{HYPO}) ($n=12$), and HPLC fractions of S_{HYPO} ($n=12$) incubated for 24 h with hepatocytes from rabbits with acute moderate hypoxia on cytochrome P450 content. Data is presented as mean±s.e.mean. *$P<0.05$ compared with S_{CONT}.

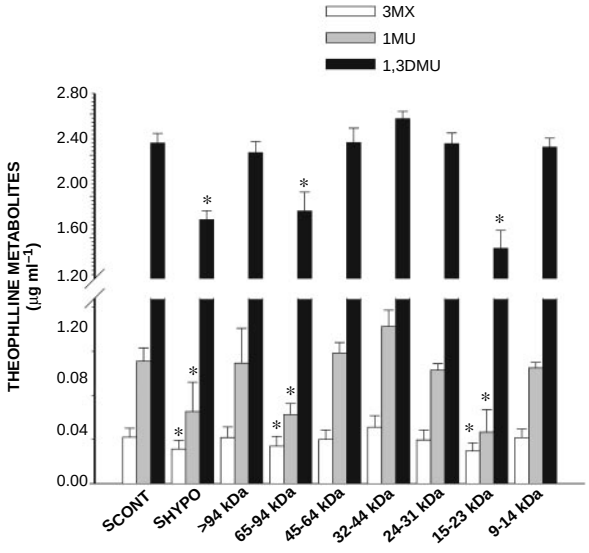

Figure 5 Effect of serum of control rabbits (S_{CONT}) ($n=10$), serum from rabbits with acute moderate hypoxia (S_{HYPO}) ($n=7$), and HPLC fractions of S_{HYPO} ($n=12$) incubated for 24 h with hepatocytes from rabbits with acute moderate hypoxia on the formation of theophylline metabolites. Data is presented as mean± s.e.mean. *$P<0.05$ compared with S_{CONT}.

The 32–44 kDa fraction of S_{HYPO} ($n=12$) increased P450 content by 33% ($P<0.05$) after 24 h of incubation with H_{HYPO}. The addition of anti-Epo antibody ($n=6$) to the 32–44 kDa fraction reduced the increase in P450 content ($P<0.05$), however, was unable to normalize it, e.g. it was still 14% greater than control ($P<0.05$) (Figure 8). On the other hand, compared to the effect of the 32–44 kDa fraction on CYP3A6 (50% increase), the addition of anti-Epo antibody ($n=6$) to the 32–44 kDa fraction reduced the increase in amount of CYP3A6 protein to 25% (Figure 10).

Discussion

In the liver, cytochrome P450 is located primarily in zone 3 or perivenous zone, where under control conditions the concentration of oxygen is the lowest (Oinonen & Lindros, 1998; Jungermann & Kietzmann, 2000). From zone 1 or periportal to the perivenous zone, the concentration of the signal oxygen falls from about 13 (arterial) to nine (mixed periportal) and then to four (hepatic venous) volume per cent gas atmosphere (Jungermann & Kietzmann, 1997). Acute moderate hypoxia depresses enzymatic activity in both periportal, such as phosphoenolpyruvate carboxykinase (Jungermann & Kietzmann, 2000) and glutathione peroxidase (Proulx & du Souich, 1995), and perivenous zone, such as CYP1A1 and 1A2 and glucuronoconjugation (Jones, 1981). This is consistent with the fact that acute anoxia produces injuries which can be more extensive in the periportal zone than in the perivenous zone (Brass *et al*., 1992). Cultured hepatocytes harvested from the entire liver without taking into account zonation should not distort the observations but could dilute the effect of hypoxia on CYP1A1, 1A2 and 3A6.

The present study demonstrates that *in vivo*, acute moderate hypoxia reduces total P450 content and activity, as well as the expression of CYP1A1 and CYP1A2, and up-regulates the expression and activity of CYP3A6. Incubation of S_{HYPO} with H_{HYPO} for 4 h diminishes the formation of theophylline metabolites without affecting total P450 content or amount of CYP1A1, 1A2 and 3A6 proteins. Moreover, incubation of S_{HYPO} with H_{HYPO} for 24 h reduces theophylline metabolites output, as well as the expression of CYP1A1 and 1A2, but increases that of CYP3A6 and its activity. On the other hand, incubation of S_{HYPO} with H_{CONT} for 4 h does not affect any of the parameters estimated, but after 24 h, reduces the output of theophylline metabolites and increases the activity of CYP3A6. These results indicate that (1) incubation of S_{HYPO} with H_{HYPO} for 24 h portrays the effect of hypoxia *in vivo*, supporting that S_{HYPO} does contain serum mediators; (2) 4 h incubations demonstrate that the down-regulation of CYP1A1 and 1A2 is preceded by a decrease in their activity; and (3) the effect of hypoxia *in vivo* is not recreated by incubating S_{HYPO} with H_{CONT}, suggesting that *in vivo* the effect of hypoxia results from an increase in circulating serum mediators and from changes in the hepatocyte.

The HPLC fractions with proteins of 15–23 kDa and 65–94 kDa M_r down-regulate CYP1A1, 1A2 and 3A6; although, in both fractions, the mediators contributing to the decrease in the expression of these isoforms are IFN-γ, IL-1β and IL-2. The efficiency and specificity of these cytokines to down-regulate CYP1A1, 1A2 and 3A6 differ. IFN-γ is not only the most potent, but also the only to down-regulate CYP1A2 and CYP3A6, whereas CYP1A1 is down-regulated by all three cytokines. These differences in efficiency and specificity explain why anti-IFN-γ antibody prevents the decrease in theophylline biotransformation, since theophylline is primarily metabolized by CYP1A2. IL-1β and IL-2 diminish theophylline metabolism because they reduce the expression of CYP1A1. Actually, individual antibodies do not prevent totally the down-regulation of CYP1A1, 1A2 and 3A6, suggesting that the effect of the serum mediators results from concerted action and/or cross-talk of several cytokines. Further supporting a concerted action of several cytokines

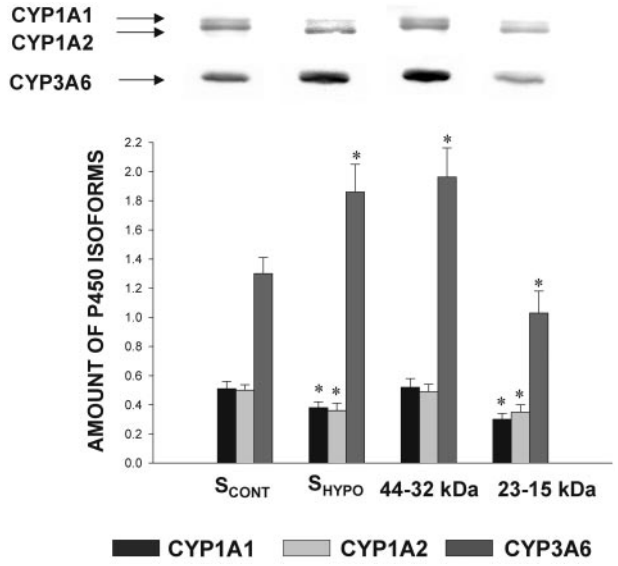

Figure 6 Effect of serum of control rabbits (S_{CONT}) ($n=10$), serum from rabbits with acute moderate hypoxia (S_{HYPO}) ($n=7$), and HPLC fractions of S_{HYPO} ($n=7$) on the amount of CYP1A1, 1A2 and 3A6 apoproteins in hepatocytes from rabbits with acute moderate hypoxia (H_{HYPO}) following 24 h incubation. Bands are representative Western blots of CYP1A1, 1A2 and 3A6. Data is presented in arbitrary units as mean ± s.e.mean. $^*P<0.05$ compared with control.

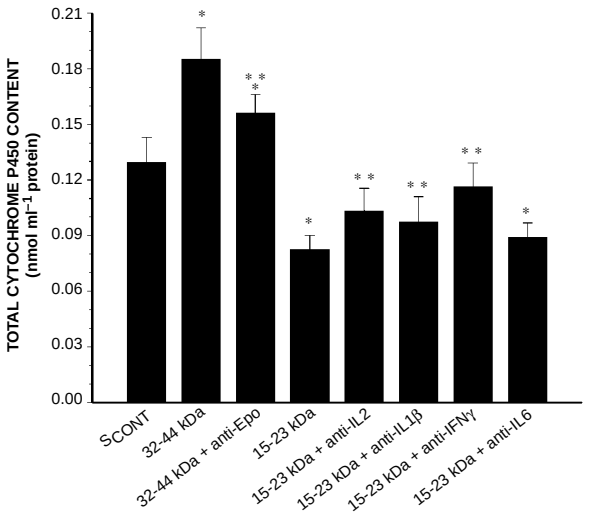

Figure 8 Effect of anti-cytokine antibodies on the ability of the 32–44 kDa and 15–23 kDa serum fractions to modify P450 content incubated for 24 h with hepatocytes from rabbits with acute moderate hypoxia. S_{CONT} is serum from control rabbits; Epo is erythropoietin; IL-2, IL-1β, IL-6 and IFN-γ are interleukin-2, -1β, -6 and interferon-γ, respectively. Data is presented as mean ± s.e.mean. $^*P<0.05$ compared with control; $^{**}P<0.05$ compared with the serum fraction without the antibody.

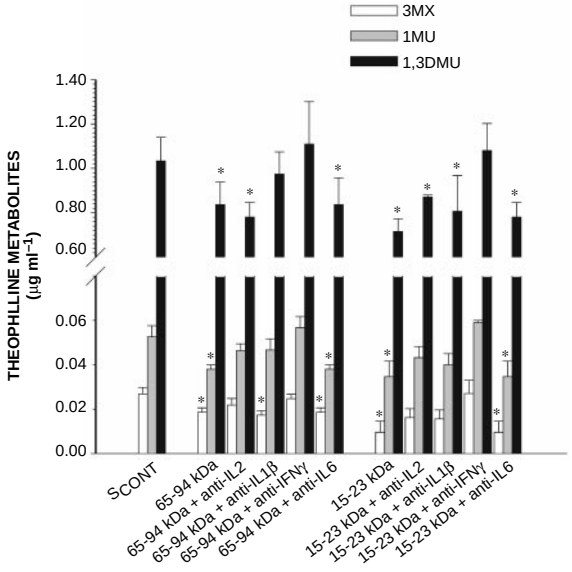

Figure 7 Effect of anti-cytokine antibodies on the ability of the 65–94 kDa and 15–23 kDa serum fractions to modify the formation of theophylline metabolites incubated for 4 h with hepatocytes from rabbits with acute moderate hypoxia. S_{CONT} is serum from control rabbits; IL-2, IL-1β, IL-6 and IFN-γ are interleukin-2, -1β and interferon-γ, respectively. Data is presented as mean ± s.e.mean. $^*P<0.05$ compared with control.

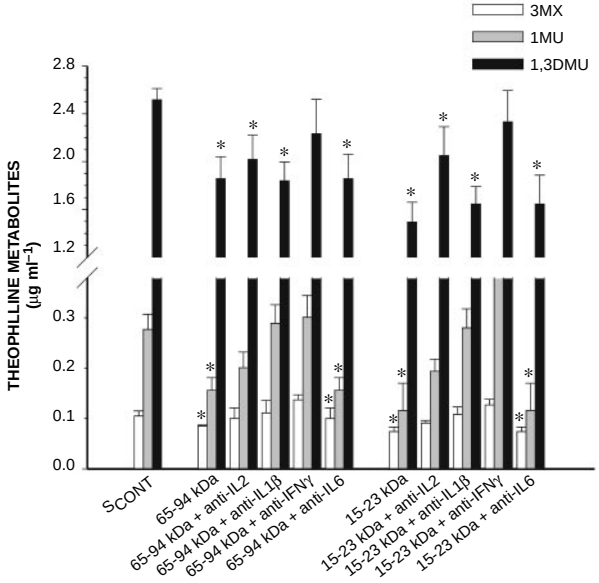

Figure 9 Effect of anti-cytokine antibodies on the ability of the 65–94 kDa and 15–23 kDa serum fractions to modify the formation of theophylline metabolites incubated for 24 h with hepatocytes from rabbits with acute moderate hypoxia. S_{CONT} is serum from control rabbits; IL-2, IL-1β, IL-6 and IFN-γ are interleukin-2, -1β, -6 and interferon-γ, respectively. Data is presented as mean ± s.e.mean. $^*P<0.05$ compared with control.

is the fact that *in vitro* experiments where individual cytokines are incubated with hepatocytes from normal animals or humans, show that IL-1β has the greatest ability to depress CYP1A1 and 1A2 isoforms (Abdel-Razzak *et al*., 1993), and that IL-2 and IL-1β depress CYP3A isoforms (Calleja *et al*., 1998). These results differ from ours and may be explained by the presence of several mediators in the serum, by differences in the concentrations of the mediators, and by differences in

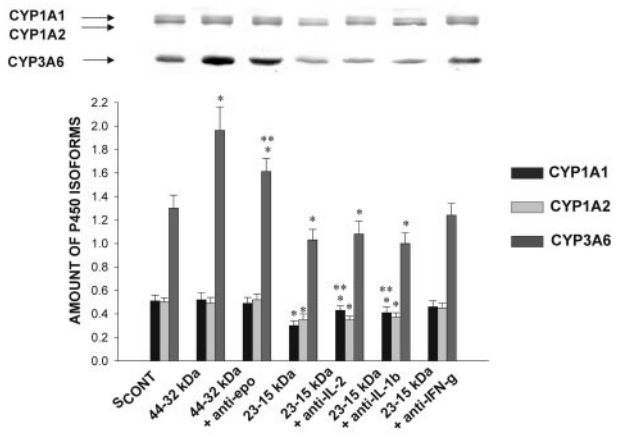

Figure 10 Effect of anti-cytokine antibodies on the ability of 32–44 kDa and 15–23 kDa serum fractions to modify the amount of CYP1A1, 1A2 and 3A6 apoproteins incubated for 24 h with hepatocytes from rabbits with acute moderate hypoxia. S_{CONT} is serum from control rabbits; Epo is erythropoietin; IL-2, IL-1β, IL-6 and IFN-γ are interleukin-2, -1β, -6 and interferon-γ, respectively. Bands are representative Western blots of CYP1A1, 1A2 and 3A6. Data is presented in arbitrary units as mean$\pm$s.e.mean. $^{*}P<0.05$ compared with control; $^{**}P<0.05$ compared with the serum fraction without the antibody.

the experimental model, e.g. control hepatocytes or hepatocytes from animals subjected to the hypoxic stress.

The fact that anti-IFN-γ, -IL-1β and -IL-2 antibodies prevented the effect of the 65–94 kDa fraction on P450 may be explained by the binding of these cytokines to plasma proteins. Effectively, IFN-γ binds to soluble receptors with M_r ranging from 45 to 67 kDa (Bello *et al*., 1998); IL-1β circulates in plasma bound to the IL-1 receptor accessory protein with a M_r of approximately 66 kDa (Greenfeder *et al*., 1995); and IL-2 can bind to a soluble receptor with a M_r of 67 kDa (Jacques *et al*., 1990). Moreover, in the case of IFN-γ, selected activities are elicited by the tetramer of 72 kDa (Langer *et al*., 1994).

Several mechanisms may have contributed to down-regulate CYP1A1 and 1A2 under hypoxic conditions. On one hand, the fact that the expression of CYP1A1/1A2 apoproteins and mRNAs decreased, supports the possibility that the effect of IFN-γ, IL-1β and IL-2 implicates a pre-translational mechanism (Morgan, 1997). On the other hand, hypoxia modifies the expression of multiple genes to improve blood delivery and cellular metabolism (Bunn & Poyton, 1996), effect in part regulated by hypoxia-inducible factor 1 transcriptional activator (HIF-1) (Semenza, 1999). HIF-1 is a basic heterodimer, with a HIF-1α subunit, unique to HIF-1, and a HIF-1β or aryl hydrocarbon receptor nuclear translocator (Arnt) subunit. HIF-1β/Arnt dimerizes also with the aryl hydrocarbon receptor (AhR) (Park, 1999). Under hypoxic conditions, HIF-1β dimerizes preferentially with HIF-1α, in such a way that less HIF-1β is available for recruitment with AhR as a heterodimeric partner. Since in adult rabbits, AhR is constitutively activated, and CYP1A1 and CYP1A2 genes are expressed constitutively (Strom *et al*., 1992), we may speculate that activation of HIF-1 by hypoxia and by S_{HYPO} reduces AhR/Arnt heterodimer and CYP1A expression (Park, 1999). This explanation may apply to H_{CONT} where S_{HYPO} decreased *CYP1A1* and *1A2* mRNA. However, S_{HYPO} did not reduce *CYP1A1* and *1A2* mRNA further in H_{HYPO}, suggesting that the decrease in CYP1A1 and 1A2 proteins is secondary to a post-transcriptional effect. The reason why S_{HYPO} reduced *CYP1A1* and *1A2* mRNA in H_{CONT} but not in H_{HYPO} remains unknown.

It is not clear how S_{HYPO} reduces the activity of CYP1A1/1A2 without affecting their amount in H_{CONT} after 4 and 24 h of incubation, and in H_{HYPO} after 4 h of incubation. We may postulate that the decrease in activity is associated with the presence of reactive oxygen intermediates (ROI). Hypoxia induces a time-dependent decrease in reduced glutathione and other cellular antioxidants, and an increase in lipid peroxidation in the liver (Proulx & du Souich, 1995), probably as a result of the formation hydrogen peroxide (H_2O_2) (Weissmann *et al*., 2000) and nitric oxide ($NO^{\bullet}$) (Gess *et al*., 1997). There is evidence that H_2O_2 and $NO^{\bullet}$ can inactivate P450 apoproteins (Karuzina & Archakov, 1994; Takemura *et al*., 1999). Indirect evidence supporting a role for ROI in P450 decrease in activity, is the fact that L-NAME, N-acetylcysteine and dimethylthiourea dose-dependently prevent the decrease in CYP1A1/1A2 activity induced by IL-6, INF-γ and IL-1β (El-Kadi *et al*., 2000).

Hypoxia *in vivo* and incubation of S_{HYPO} with H_{HYPO} *in vitro* increase the expression of CYP3A6 and *CYP3A6* mRNA. The expression of CYP3A4 is modulated by nuclear receptors, such as constitutive androstane receptor (CAR), pregnane X receptor (PXR), retinoid X receptor (RXR), hepatocyte nuclear factor 4 (HNF-4), and glucocorticoid receptor (GR) (El-Sankary *et al*., 2000). Assuming that CYP3A6 is modulated as CYP3A4, since hypoxia activates HIF-1 in cooperation with HNF-4, RXR, and GR (Bunn *et al*., 1998; Machein *et al*., 1999; Kambe *et al*., 2000), we may speculate that these proteins are implicated in the increase in *CYP3A6* gene and CYP3A6 apoprotein. The increase in expression of CYP3A6 by the 32–44 kDa fraction is partially reversed by anti-Epo antibodies. There is no data supporting that Epo modulates directly the expression of CYP3A. On the other hand, Epo binding to its membrane receptor activates phosphatidylinositol 3-kinase (PI3-K) (Gaffen *et al*., 1999; Lacombe & Mayeux, 1999), which is required for HIF-1 stabilization and translocation (Mazure *et al*., 1997). We postulate that Epo facilitates HIF-1 cooperation with nuclear receptors to enhance CYP3A6 expression.

The increase in CYP3A6 induced by S_{HYPO} was greater in H_{HYPO} than in H_{CONT}, suggesting that serum mediators elicit a greater effect in primed hepatocytes. Several mechanisms, not mutually exclusive, are plausible to prime the hepatocyte: (a) hypoxia increases the expression of the nuclear factors RXR, HNF-4, and GR (Machein *et al*., 1999; Kambe *et al*., 2000); (b) hypoxia modulates protein tyrosine kinase (Uchiyama *et al*., 2000), AMP-activated protein kinase Blazquez *et al*., 1999), and protein kinase C (Hsu & Huang, 1998) activities, known to regulate the expression of CYP3A (Brown *et al*., 1997); (c) hypoxia activates PI-3K required for HIF-1 stabilization and translocation (Mazure *et al*., 1997); and (d) hypoxia increases plasma levels of cytokines which enhance the density of cytokines surface receptors (Simms & D'Amico, 1996; Takabatake *et al*., 2000). Any of these mechanisms could contribute to increase responsiveness of H_{HYPO} by comparison with H_{CONT}.

In conclusion, the present study demonstrates that acute moderate hypoxia modifies the expression of hepatic P450 isoforms by several mechanisms, including serum mediators and intracellular adaptation to hypoxia. Hypoxia induces the release of serum mediators, among them IFN-γ, IL-1β and IL-2, that reduce initially the activity and thereafter decrease the expression of CYP1A1 and 1A2 and of *CYP1A1* and *1A2* genes. In addition, acute moderate hypoxia increases the expression of CYP3A6 protein and *CYP3A6* gene, effect partially mediated by circulating Epo. The pathophysiological role of CYP3A6 induction is unknown. Intracellular adaptation to hypoxia is evidenced by the fact that the combined effect of all these mediators reproduce the effects observed *in vivo* when they are incubated with H_{HYPO} but not with H_{CONT}.

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