Animal Models of Acute Moderate Hypoxia Are Associated with a Down-Regulation of CYP1A1, 1A2, 2B4, 2C5, and 2C16 and Up-Regulation of CYP3A6 and P-glycoprotein in Liver

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ABSTRACT:

In humans, indirect evidence suggests that hypoxia reduces the rate of biotransformation of drugs cleared by cytochrome P450 (P450) subfamilies CYP1A, 2B, and 2C. The aim of this study was to assess whether acute moderate hypoxia modulates the expression of CYP2B4, 2C5, and 2C16 in vivo, and to determine whether the changes in hepatic P450 are conveyed by serum mediators. Moreover, because hypoxia increases the expression of P-glycoprotein in vitro, we examined whether in vivo acute moderate hypoxia modulates the expression of several membrane transporters in the liver. Rabbits and rats were exposed to a fractional concentration of oxygen of 8% for 48 h to generate a stable arterial partial pressure of O₂ of 34 ± 1 mm Hg. Compared with rabbits breathing room air, hypoxia in rabbits reduced the amount of CYP1A1, 1A2, 2B4, 2C5, and 2C16 proteins and increased the expression of CYP3A6. Sera of rabbits with hypoxia were fractionated by size exclusion chromatography, the fractions were tested for their ability to modify the expression of P450 isoforms, and serum mediators were identified through neutralization experiments. The serum mediators responsible for the down-regulation of P450 isoforms were interferon-γ, interleukin-1β (IL-1β), and IL-2. In vivo, rats, hypoxia increased the mRNA and protein expression of P-glycoprotein but did not affect the mRNA of breast cancer resistance protein and organic anion-transporting polypeptide 2. It is concluded that in vivo, hypoxia down-regulates rabbit hepatic CYP1A1, 1A2, 2B4, 2C5, and 2C16 and up-regulates CYP3A6. CYP3A11 and P-glycoprotein were up-regulated in the livers of hypoxic rats.

In patients with cardiorespiratory diseases, acute hypoxemia appears to reduce the ability of cytochrome P450 isoforms to catalyze the biotransformation of xenobiotics. Patients with pulmonary insufficiency who show an impairment of their general state present an increased incidence of adverse effects, e.g., grand mal seizures, associated with the administration of “usual” doses of theophylline. Theophylline clearance is significantly reduced in patients with acute cardiogenic pulmonary edema (Piafsky et al., 1977) and in patients with worsening airway obstruction, severe bronchial obstruction, congestive heart failure, and pneumonia (Vozeh et al., 1978).

In humans, at therapeutic concentrations, the biotransformation of theophylline is primarily catalyzed by CYP1A2, whereas CYP2D6, 2E1, and 3A4 exhibit low affinity and variable capacity (Ha et al., 1995). Rabbits exposed to a fractional concentration of inspired O₂ (FiO₂) of 10% for 24 h demonstrate a reduced clearance of theophylline and decreased expression of CYP1A1 and 1A2, although the expression of CYP3A6 is increased (Kurdi et al., 1999). In rabbits subjected to acute moderate hypoxia, hepatic down-regulation of CYP1A1 and 1A2 is triggered by serum mediators, e.g., interferon-γ (IFN-γ), interleukin 2 (IL-2), and IL-1β; the up-regulation of CYP3A6 is, at least in part, associated with erythropoietin (Epo) (Fradette et al., 2002). In vivo, the increase in CYP3A6 by hypoxia is associated with the increased expression and nuclear translocation of hypoxia-inducible factor-1 (HIF-1), activator protein 1 (AP-1), and constitutive androstane receptor (Fradette and du Souich, 2003).

The expression of CYP3A6 is, at least in part, associated with erythropoietin (Epo) (Fradette et al., 2002). In vivo, the increase in CYP3A6 by hypoxia is associated with the increased expression and nuclear translocation of hypoxia-inducible factor-1 (HIF-1), activator protein 1 (AP-1), and constitutive androstane receptor (Fradette and du Souich, 2003).

Abbreviations: FiO₂, fractional concentration of inspired O₂; AhR, aryl hydrocarbon receptor; AP-1, activator protein-1; Arnt, aryl hydrocarbon receptor nuclear translocator; BCRP, breast cancer resistance protein; Epo, erythropoietin; HCONT, hepatocytes from control rabbits; HYPO, hepatocytes from rabbits with hypoxia; HIF-1, hypoxia-inducible factor 1 transcriptional activator; HPLC, high performance liquid chromatography; IFN-γ, interferon-γ; IL, interleukin; MDR1, multidrug resistance protein 1; Mr, relative molecular mass; NK-κB, nuclear factor κB; OATP2, organic anion transporting polypeptide 2; P450, cytochrome P450; PXR, pregnane X receptor; SCONT, serum from control rabbits; SHYPO, serum from rabbits with hypoxia.
The present study aimed to explore whether acute moderate hypoxia down-regulates CYP2B4, 2C5, and 2C16 in vivo, the rabbit’s orthologs of human CYP2B6, 2C9 and 2C19, respectively, because of their relative importance in the biotransformation of drugs, and to identify ex vivo the serum mediators implicated in the in vivo down-regulation of these hepatic isozymes. In contrast, P-glycoprotein is modulated by constitutive androstane receptor (Burk et al., 2005) and by the presence of reactive oxygen species (Wartenberg et al., 2003), which are increased by acute moderate hypoxia (Fradette and Du Souich, 2004); moreover, the multidrug resistance protein 1 (MDR1) gene contains a binding site for HIF-1 (Wartenberg et al., 2003). Thus, we hypothesized that in vivo, hypoxia would increase the expression of the P-glycoprotein MDR1 (multidrug resistance gene product, ABCB1). Therefore, the present study also aimed to document in vivo the effect of moderate hypoxia on several efflux and influx membrane transporters.

Materials and Methods

Animals and Collection of Hepatocytes. Male New Zealand White rabbits (1.8–2.2 kg) were obtained from Ferme Charles Rivers (St. Constant, QC, 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 hypoxia, rabbits were placed in a Plexiglas chamber (0.75 × 1.20 × 1.25 m²) with a FiO₂ of 8%, adjusted with an oxygen monitor (OM-15; Valves, Brantford, ON, Canada), which uses nitrogen to displace oxygen from the chamber. This FiO₂ generates a stable arterial partial pressure of O₂ of approximately 35 mm Hg (Fradette et al., 2002; Fradette and du Souich, 2003). Control rabbits were placed into the Plexiglas chamber containing room air (FiO₂ = 21%) for the experiments. All the rabbits remained in the chamber for 48 h where they had access to Purina Laboratory Chow and water ad libitum. All the experiments were conducted according to the Canadian Council on Animal Care guidelines for use of laboratory animals.

Hepatocytes from rabbits with hypoxia (HHYPO) and from control rabbits (HCONT) were isolated 48 h after the induction of hypoxia or breathing room air, respectively, according to the two-step liver perfusion method with minor modifications (El-Kadi et al., 1997). Rabbits were anesthetized with 30 mg/kg sodium pentobarbital, and after a middle laparotomy, the portal and inferior cava veins were cannulated. The liver was perfused in situ via the portal vein with a washing solution: 115 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 25 mM HEPES, 0.5 mM EGTA, 5.5 mM glucose, and 56.8 mg/ml heparin, followed by a perfusion of a solution of 0.013% collagenase, 1 mM CaCl₂, and 0.25 mM trypsin inhibitor. Harvested cells were centrifuged on a 40% Percoll gradient to isolate viable hepatocytes. Cell cultures were always conducted under sterile conditions. Viability was assessed by trypan blue exclusion to ensure that it was greater than 90%; viability was not affected by in vivo hypoxia or any other experimental condition. Cell concentration was adjusted to 1 × 10⁶ cells per ml with Williams’ medium E supplemented with 10% calf serum and 1 µM insulin. Aliquots of 2 ml of the hepatocytes in suspension were transferred into six-well plastic culture plates (Falcon; BD Biosciences Discovery Labware, Bedford, MA) coated with type 1 rat-tail collagen and incubated for 2 h at 37°C in an atmosphere of 95% air and 5% CO₂ to allow stabilization before any experiment was undertaken.

Characterization of Serum Mediators. 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 tube (BD Biosciences, Mississauga, ON, Canada). Blood samples were allowed to clot at room temperature for 2 h and centrifuged thereafter at 2500 rpm for 5 min; then, 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. Preliminary studies have shown that when samples were handled as described, serum mediators conserved their activity for up to 12 months. Serum proteins in the serum were separated by size exclusion high performance liquid chromatography (HPLC) on a Superose 12 HR column from GE Healthcare BioSciences Inc. (Baie d’Urfe, QC, Canada) as described previously (Fradette et al., 2002). In brief, the flow rate of the mobile phase was set at 0.3 ml min⁻¹ and column pressure was maintained between 9 and 12 bar with an LKB (Bromma, Sweden) 2150 HPLC pump. Absorbance was measured at 280 nm with a Waters 490E spectrophotometric detector (Millipore, Billerica, MA). The eluant buffer included 115 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1 mM HEPES, 25 mM EGTA, and 5.5 mM glucose; the pH of the solution was adjusted to 7.4 and filtered through a 0.22-µm membrane. Serum aliquots of 300 µl were injected into the column and fractions of 1.2 ml were collected with a fraction collector (LKB 2211 Supercarr). To calculate the Mᵣ 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): 1-glutamic dehydrogenase (55,600), aldolase (39,200), triosephosphate isomerase (26,600), trypsin inhibitor (26,600), cytochrome c (12,500), and aprotinin (65,000). The fractions containing proteins with Mᵣ between 9000 and 14,000, 15,000 and 23,000, 24,000 and 31,000, 32,000 and 44,000, 45,000 and 64,000, and 65,000 and 94,000 were separated and collected. Based on the size exclusion range of the column, proteins isolated in the first fraction were assumed to have a Mᵣ greater than 95,000. To increase the inhibitory activity of the fractions collected, these were concentrated on Microsep 3K membranes ( Pall Filtron, Northborough, MA) that retain proteins greater than 3000. Three milliliters of the fractions were added to the sample reservoir and centrifuged at 75,000 g to reduce the volume and hence concentrate serum fractions 1.25 times.

The medium of the cultured hepatocytes was replaced with fresh Williams’ medium E supplemented with 10% calf serum and 1 µM insulin, and hepatocytes (HCONT and HHYPO) were then incubated with 200 µl of serum from control rabbits (SCONT), 200 µl of serum from hypoxic rabbits (SHYPO), 200 µl of the HPLC fractions of SCONT and SHYPO, or 200 µl of saline, i.e., in the absence of serum or its fractions, for 24 h. After 24 h of incubation, the effect of serum or the HPLC fractions was assessed by measuring the expression of CYP1A1/1A2, 2B4, 2C5, 2C16, and 3A6.

The identification of the serum mediators was performed by immunoneutralization. The selection of the antibodies used for the immunoneutralization of cytokines was based 1) upon the Mᵣ of the proteins incorporated in the HPLC fractions having the ability to change P450 isoform expression, and 2) according to the cytokines released during hypoxia (Naldini et al., 1997). To prevent the changes in P450 isoform expression induced by the HPLC fractions, the following antibodies were used: a goat anti-rabbit IL-1β (anti-IL-1β) polyclonal antibody, and anti-human IL-2 (anti-IL-2), IFN-γ (anti-IFN-γ), IL-6 (anti-IL-6), and Epo (anti-Epo) monoclonal antibodies. The antibodies against human proteins were used to neutralize the homologous rabbit proteins because of the known interspecies reactivity of these antibodies (Fradette et al., 2002). An IgG monoclonal antibody to Pseudomonas aeruginosa served as control. Aliquots of 2 µg of each antibody were added individually to 200 µl of the HPLC fractions that were able to decrease the expression of P450 isoforms, and were incubated at 37°C for 1 h. The antibody and the HPLC fractions were incubated with the hepatocytes for 24 h when P450 isoform expression was assessed. The amount of 2 µg of antibodies was used because this amount is effective to immunoneutralize IL-1β, IL-6, and IFN-γ in the sera of rabbits and of humans with an inflammatory reaction (Fradette et al., 2002).

Measure of P450 Isoforms by Immunoblot. Hepatocytes were washed, harvested in ice-cold phosphate-buffered saline, and centrifuged at 1500g for 5 min. The pellet was resuspended in ice-cold lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM diethiothreitol, protease inhibitor mixture), and cells were allowed to swell on ice for 15 min and vortexed for 30 s. The amount of protein in hepatocytes was measured in cell lysate by the method of Lowry et al. (1951). For Western blot analysis, 50 µg of cell lysate were separated by SDS-polyacrylamide gel electrophoresis (Fradette et al., 2002). Separated proteins were electrophoretically transferred to a nitrocellulose membrane using a semidyke transfer process (Bio-Rad, Hercules, CA). CYP1A1 and 1A2 were detected with a polyclonal anti-rabbit CYP1A1 (Oxford Biomedical Research, Oxford, MI) diluted 1:100 in 5% nonfat milk in Tris-buffered saline/Tween 20 (0.1% Tween 20) and visualized with an alkaline phosphatase-conjugated secondary antibody using nitro blue tetraazolium as substrate. CYP2B4, 2C5, 2C16, and 3A6 antibodies were detected with rabbit anti-human antibodies, which recognize rabbit isoforms, against CYP2B4 diluted 1:500 (Research Diagnostics, Flanders, NJ), CYP2C5 diluted 1:100 (Research Diagnostics), CYP2C16 diluted 1:100 (Research Diagnostics), and CYP3A6 diluted 1:500 (Oxford Biomedical Research), using a...
secondary antibody conjugated with a chemiluminescence reagent (horseradish peroxidase enzyme) and visualized by autoradiography. As reference protein, in each gel, 50 μg of proteins extracted from the same control hepatocytes, with constant amounts of CYP1A1, 1A2, 2B4, 2C5, 2C16, and 3A6, were used. The assays were linear in the range of protein amounts assessed under the actual experimental conditions, and the results are presented as a ratio of the P450 isoform to the reference protein.

**Effect of Hypoxia on the Expression of Membrane Transporters.** These experiments were conducted with rats because of the lack of information and commercial availability of antibodies for transporters in rabbit. Preliminary experiments showed that a FiO2 of 8% reduced the arterial partial pressure to levels similar to those measured in rabbits, i.e., around 35 mm Hg, and that after 48 h, the expression of CYP3A1 was induced consistent with that seen in rabbits.

Male Sprague-Dawley rats (250–275 g) were purchased from Charles River Canada (St. Constant, QC, Canada), and studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Rats were exposed for 48 h to a FiO2 of 8% in the chamber as described above. Control rats were placed into chambers that contained room air (FiO2 = 21%) for the experiments. Forty-eight hours later, the rats were anesthetized with halothane and the liver was removed, rapidly frozen in liquid N2, and stored at -80°C until used for protein and RNA isolation. The expression of CYP3A1 was measured from the microsomal fraction of the liver as described above. To further validate the rat model, preliminary experiments showed that the effect of 48 h of hypoxia in vivo on the expression of P450 isoforms was not affected by the approach used, e.g., measure of P450 isoforms in microsomes or in cultured hepatocytes.

**Analysis of Mdr, OATP, BCRP, and PXR Expression.** Total RNA was isolated from rat liver using the QuickPrep RNA extraction kit (GE Healthcare BioSciences Inc.) according to the manufacturer’s protocol, and cDNA was synthesized from 2.5 μg of RNA using the First Strand cDNA Synthesis kit (MBI Fermentas, Flamborough, ON, Canada). Transporter mRNA levels were measured by real-time quantitative polymerase chain reaction using LightCycler technology (Roche Diagnostics, Mannheim, Germany) with LC FastStart DNA Master SYBR Green I for detection. Primer sequences for Mdr1a, Mdr1b, and PXR were previously reported (Kaliszky-Szirtes et al., 2004). Primers for breast cancer resistance protein (BCRP) were forward, 5'-AGTC- CCGAATACAGCTGGA-3', and reverse, 5'-CCCATCAACAGCT- CATCTTG-3'; and for OATP2 were: forward, 5'-TTGTTGTTGATGTTG- CAG TT-3', and reverse, 5'-GCCAATGGTACCTCCTGTT-3'. All mRNA levels were normalized to 18S mRNA and the ratios are presented as percent.

The hepatic crude membrane fraction was isolated as described previously (Teng and Piquette-Miller, 2005) and measured by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay. For the immunoblotting, 10 μg of protein was loaded and run on a 10% acrylamide gel and transferred onto a Hybond ECL nitrocellulose membrane (GE Healthcare BioSciences Inc.). Membranes containing transporter protein were then cut in half and the upper portion (mol. wt. >78,000) was incubated overnight with 2 μg of the P-glycoprotein monoclonal antibody, C219 (BD Labs Inc., London, ON, Canada), followed by sheep anti-mouse IgG (Amersham). To control for variability in protein loading, the lower portion of the membrane (mol. wt. <78,000) was incubated with anti-β-actin clone AC15 antibody (Sigma-Aldrich, St. Louis, MO) followed by sheep anti-mouse IgG (GE Healthcare BioSciences Inc.). Bands were detected using an ECL Western blotting analysis system (GE Healthcare BioSciences Inc.), imaged on Bioflex MSi film (Clonex Corp. and InterSciences Inc., Markham, ON, Canada), and quantified using Kodak Digital Science 1D Image Analysis software (Eastman Kodak, Rochester, NY).

**Drugs and Chemicals.** Percoll gradient, Williams’ medium E, calf serum, type I rat-tail collagen, NaCl, KCl, KH2PO4, Heps, EGTA, and glucose were purchased from Sigma Chemical (St. Louis, MO). Insulin was acquired from Roche Diagnostics (Laval, QC, Canada), and L-glutamic dehydrogenase, aldolase triosephosphate isomerase, tryosin inhibitor, cytochrome c, and aprotinin were obtained from GE Healthcare BioSciences Inc.). The polyclonal anti-rabbit CYP1A1 and the monoclonal anti-rat CYP3A1 antibodies were purchased from Oxford Biomedical Research; the anti-CYP2B4, anti-CYP2C5, and anti-CYP2C16 antibodies from Research Diagnostics; the anti-IL-1β antibody from Cedar Lane (Hornby, ON, Canada); and the anti-IL-2, anti-IL-6, and anti-IFN-γ antibodies from R&D Systems (Minneapolis, MN).

**Statistical Analysis.** All results are presented as mean ± S.E. The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one-way analysis of variance, 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 Isoform Expression.** Exposure of rabbits to an 8% FiO2 atmosphere for 48 h generated a stable hypoxemia, with an average PaO2 of 34 ± 1 mm Hg, without influencing the arterial partial pressure of CO2 (≈21 ± 1) and arterial pH (7.47 ± 0.05). Compared with rabbits breathing a 21% FiO2 (i.e., room air), hypoxia reduced the amount of CYP1A1, 1A2, 2B4, 2C5, and 2C16 proteins by 37, 40, 55, 75, and 82%, respectively (p < 0.05, n = 6 per group). On the other hand, 48 h of hypoxia increased the amount of CYP3A6 by 71% (Fig. 1).

**Effect of SCONT and SHYPO on Hepatic P450 Isoform Expression in HCONT and in HHYPO**. Compared with HCONT incubated with SCONT, incubation of HHYPO with SHYPO for 24 h reduced the amount of CYP2C5 and 2C16 by 15% and 17%, respectively (p < 0.05). Moreover, SHYPO increased the amount of CYP3A6 by 31% (p < 0.05, n = 6). No changes in CYP1A1, 1A2 and 2B4 proteins were observed (Fig. 2A).

In HHYPO incubated with SCONT, the expression of CYP1A1, 1A2, 2B4, 2C5, and 2C16 was reduced by 33, 35, 42, 70, and 77%, respectively (p < 0.05, n = 6), and that of CYP3A6 was increased by approximately 70% (p < 0.05), compared with HCONT (Fig. 2B). After 24 h of incubation of HHYPO with SHYPO, the amount of CYP1A1 and 1A2 proteins was further reduced by an average of 27 and 30%, respectively (p < 0.05), whereas the amount of CYP2B4, 2C5, and 2C16 decreased to an undetectable level. On the other hand, incubation of HHYPO with SHYPO increased the expression of CYP3A6 by an average of 50% (p < 0.05) (Fig. 2B).

**Effect of SHYPO, HPLC Fractions on P450 Isoform Expression in HHYPO.** Compared with the SCONT, incubation of the SHYPO 15- to
densitometric ratios.

CYP1A1 and 2C19 (Addition of an anti-IL-2 antibody partially prevented the decrease of bation of these sera with several antibodies influenced this change.

SCONT incubated with HCONT. Data are mean n/H11005

bations of SCONT fractions with HCONT did not modify the expression of CYP1A1, 1A2, 2B4, 2C5, 2C16, and 3A6 (data not shown).

rabbits (A; HCONT, SHYPO HPLC fractions did not affect the expression of any of the CYP1A1, 1A2, 2B4, 2C5, 2C16, and 3A6. The anti-IL-6 antibody CYP3A6 protein by 52% (23-kDa HPLC fraction with HHYPO for 24 h decreased expression of CYP3A6 through a protein tyrosine kinase- and an extracel-

Identification of the Mediators in SHYPO Responsible for the Changes in the Expression of P450 Isoforms. Compared with incubation with the SHYPO 15- to 23-kDa fraction, which significantly increased the expression of CYP3A6 through a protein tyrosine kinase- and an extracellular signal-regulated kinase 1/2-dependent activation of HIF-1 and dular signal-regulated kinase 1/2-dependent activation of HIF-1 and

Discussion

The present study demonstrates that besides CYP1A1 and 1A2, acute moderate hypoxia down-regulates in vivo CYP2B4, 2C5, and 2C16, the orthologs of human CYP2B6, 2C9, and 2C19, respectively. The serum mediators contributing to the decrease in expression of these isoforms are IFN-

23-kDa HPLC fraction with HHYPO, for 24 h decreased expression of CYP1A1, 1A2, and 3A6 proteins by 42, 30, and 21%, respectively (p < 0.05, n = 6), whereas CYP2B4, 2C5 and 2C16 were reduced to undetectable levels (Fig. 3). On the other hand, incubation of HHYPO with the SHYPO 32- to 44-kDa fraction increased the expression of CYP3A6 protein by 52% (p < 0.05, n = 7) (Fig. 3). The remaining SHYPO HPLC fractions did not affect the expression of any of the P450 isoforms studied compared with SCONT. Likewise, 24-h incubations of SCONT fractions with HCONT did not modify the expression of CYP1A1, 1A2, 2B4, 2C5, 2C16, and 3A6 (data not shown).

Identification of the Mediators in SHYPO Responsible for the Changes in the Expression of P450 Isoforms. Compared with the incubation with the SHYPO 15- to 23-kDa fraction, which significantly reduced the expression of all P450 isoforms in HYPO cells, coincubation of these sera with several antibodies influenced this change. Addition of an anti-IL-2 antibody partially prevented the decrease of CYP1A1 and 2C19 (n = 6); anti-IL-1β antibody partially attenuated the down-regulation of CYP1A1, 2C5, 2C16 (n = 6); and, finally, anti-IFN-γ antibody (n = 6) prevented the down-regulation of CYP1A1, 1A2, 2B4, 2C5, 2C16, and 3A6. The anti-IL-6 antibody (n = 6) did not elicit any effect on P450 isoform expression, despite increasing the amount of antibody to 4 μg (data not shown).

Whereas the SHYPO 32- to 44-kDa fraction significantly increased CYP3A6 expression by approximately 80%, coincubation of an anti-

Effect of Moderate Hypoxia on the Expression of Membrane Transporters. Compared with control rats (n = 6), the expression of CYP3A1 increased by 75% (p < 0.05, n = 9) in hypoxic rats exposed to a FiO2 of 8% for 48 h (Fig. 5). Levels of CYP3A1 mRNA were also similarly induced in hypoxic rats (data not shown). Levels of mdr1b mRNA were significantly induced in hypoxic rats (p < 0.05, n = 9); however, hypoxia did not affect the expression of Mdr1a, OATP2, and BCRP mRNAs (Fig. 6). Likewise, protein levels of P-glycoprotein were significantly increased by 77% in hypoxic rats (p < 0.05, n = 9) (Fig. 5). Hypoxia tended to increase PXR mRNA (p > 0.05, n = 9) (Fig. 6).

The cytokines modulating P450 isoforms depend upon the experimental condition. For instance, in hypoxia, IL-6 does not contribute to the decrease in expression of P450 isoforms, contrasting with the turpen-

Fig. 2. Effect of serum from rabbits on the expression of cytochrome P450 isoforms. SCONT and SHYPO were incubated for 24 h with hepatocytes from control rabbits (A; HCONT, n = 6) and from rabbits with acute moderate hypoxia (B; HHYPO, n = 6), and the amounts of CYP1A1, 1A2, 2B4, 2C5, 2C16, and 3A6 apoproteins were measured in hepatocytes by immunoblot. Data are mean ± standard error of densitometric ratios. *, p < 0.05 compared with SCONT; **, p < 0.05 compared with SCONT incubated with HCONT.

Fig. 3. Effect of serum and its HPLC fractions on the expression of cytochrome P450 isoforms. SCONT, SHYPO, and the HPLC serum fractions of 23 to 15 kDa and 44 to 32 kDa were incubated for 24 h with hepatocytes from rabbits with acute moderate hypoxia (n = 6–7), and the amount of CYP1A1, 1A2, 2B4, 2C5, 2C16 and 3A6 apoproteins was measured in hepatocytes by immunoblot. Data are mean ± standard error of densitometric ratios. *, p < 0.05 compared with SCONT.
tine-induced inflammatory reaction, where IL-6 is the most important serum mediator involved in the down-regulation of CYP1A1, 1A2, and 3A6 (Bleau et al., 2003). Moreover, IFN-γ is the predominant mediator in acute moderate hypoxia and in the serum from humans with an upper respiratory viral infection, whereas IFN-γ does not modulate the expression of P450 isoforms in the turpentine-induced inflammatory reaction (Bleau et al., 2003). IFN-γ does not appear to be selective since it contributes to the down-regulation of all P450 isoforms investigated. On the other hand, in the serum of rabbits with hypoxia, IL-1β contributes to the down-regulation of CYP1A1, 2B4, and 2C5, whereas in the serum of rabbits with a turpentine-induced inflammatory reaction, IL-1β reduces CYP1A2 and 3A6, and in the serum from humans with an upper respiratory viral infection, IL-1β diminishes only CYP3A6. Moreover, the effect of serum from hypoxic rabbits depends upon the source of hepatocytes; for example, the down-regulation of CYP1A1, 1A2, 2B4, 2C5, and 2C16 and the up-regulation of CYP3A6 after 24 h of incubation with serum from rabbits with hypoxia is marginal in hepatocytes from control rabbits but extensive in hepatocytes from rabbits with hypoxia. Similar differences occur when serum from rabbits with an aseptic inflammatory reaction is incubated with hepatocytes from control rabbits or with hepatocytes from rabbits with an inflammatory reaction. The differences in response to serum may be associated with the expression of cytokine membrane receptors, which is enhanced by increased serum concentrations of cytokines secondary to the inflammatory reaction and hypoxia (Geisterfer et al., 1993; Naldini et al., 1997).

Considering CYP1A1 and 1A2, at least three mechanisms may contribute to their decrease in expression by hypoxia. In vivo, hypoxia induces the production of reactive oxygen species in the liver (Fradette and Du Souich, 2004), which may activate nuclear factor-κB (NF-κB), which can, in turn, interact with the aryl hydrocarbon receptor (AhR). This leads to a mutual functional repression of AhR by NF-κB and, consequently, to a reduction of the expression of CYP1A1 and CYP1A2 (Tian et al., 1999).

Hypoxia also activates transcription factors that regulate adaptive responses in cells (Semenza, 2000). Cells adapt to low partial pressure of oxygen by up-regulating the transcription of multiple genes, such as vascular endothelial growth factor, Epo, and several glycolytic en-FIG. 5. Effect of hypoxia on CYP3A1 expression in rat liver. Rats were exposed to room air (control) or to a FiO2 of 8% (hypoxic) for 48 h. The liver was harvested, and CYP3A1 was measured by immunoblot and normalized to its reference protein control as described under Materials and Methods. Data are presented as mean ± standard error of densitometric ratios in control (n = 6) and hypoxic (n = 9) rats (* p < 0.05). Representative blots demonstrate CYP3A1 levels in three hypoxic and three control rats.

FIG. 6. Effect of hypoxia on transporter and PXR mRNA expression in rat liver. Rats were exposed to room air (control) or to a FiO2 of 8% (hypoxic) for 48 h. The liver was harvested and total RNA was extracted. The mRNA levels of Mdr1a, Mdr1b, OATP2, BCRP, and PXR were measured by real-time quantitative polymerase chain reaction and normalized to levels of 18S mRNA as described under Materials and Methods. Bar graphs represent the mean ± standard error in control (n = 6) and hypoxic (n = 9) rats (*, p < 0.05 versus controls).
zymes. These target genes are induced by a common transcription factor, HIF-1 (Aragones et al., 2001). Hypoxia, cytokines, and ROI stabilize HIF-1α, which translocates to the nucleus, where it dimerizes with HIF-1β or aryl hydrocarbon receptor nuclear translocator (Arnt) to form HIF-1. Because Arnt is also a heterodimerization partner of AhR, hypoxia will decrease the availability of Arnt and so cause a down-regulation of CYP1A1 and 1A2 (Chan et al., 1999). Supporting the idea that hypoxia reduces CYP1A1 and 1A2 by decreasing the availability of Arnt is the fact that stabilization of HIF-1α with cobalt chloride decreases CYP1A1 and 1A2 expression (Fradette and Du Souich, 2003).

AP-1 is activated by hypoxia by the increase in intracellular calcium. Hypoxia increases intracellular calcium, which activates extracellular signal-regulated kinase 1/2 with the subsequent induction of the immediate-early response genes c-fos, c-jun, and erg-1 (Michiels et al., 2000). Dimerization of these early response genes forms AP-1, which binds to DNA to modulate gene transcription. CYP1A1 and 1A2 negative regulatory element contains AP-1 motifs (Chung and Bresnick, 1997). There is evidence that CYP1A1 down-regulation by IL-1β and IFN-γ depends upon the activation of AP-1 (Abdel-Razzak et al., 1994). On the other hand, we have shown that activation of AP-1 by lead acetate increases its nuclear translocation as well as the down-regulation of CYP1A1 and CYP1A2 in hepatocytes (Fradette and du Souich, 2003). All these data suggest that AP-1 could contribute to the CYP1A1 and 1A2 down-regulation.

Hypoxia promotes the release of cytokines including IL-1β, IL-2, IL-4, IL-5, IL-6, tumor necrosis factor α, and IFN-γ (Naldini et al., 1997). The increase in IL-1β, IL-2, and IFN-γ activates transcription factors, such as NF-κB, AP-1, and c-myc (Wesselingborg et al., 1997; Ellery and Nicholls, 2002). There is evidence that in response to IL-1β, the p50 subunit of NF-κB binds to the negative κB response element 1 of the CYP2C11 gene, entailing a down-regulation of CYP2C11 (Iber et al., 2000). Moreover, c-myc diminishes the expression of P450 isoforms by decreasing CCAAT-enhancer binding protein-α-mediated P450 transactivation (Tinel et al., 2003). Therefore, multiple mechanisms probably contribute to the modulation of CYP1A1, 1A2, 2B4, 2C5, and 2C16 expression during hypoxia.

The present results afford a mechanism to early reports showing that in humans and animals, hypoxia modulates the clearance of drugs. For instance, antipyrine half-life is increased in hypoxic subjects (Cumming, 1976), possibly because antipyrine is biotransformed by CYP1A2, 2B6, 2C8, 2C9, 2C18, and 3A4 (Engel et al., 1996). Acute moderate hypoxia reduces the clearance of phenytoin, which is biotransformed primarily by CYP2C9 and to a minor degree by CYP2C19 (Giancarlo et al., 2001). On the other hand, the clearance of lidocaine and diltiazem, two drugs primarily metabolized by CYP3A6 in rabbits, is not affected by hypoxia (Fradette and Du Souich, 2004).

The present results demonstrate that in vivo, acute moderate hypoxia increases the expression of the efflux transporter P-glycoprotein in hepatocytes as well as Mdr1b mRNA. These results confirm in vitro results showing that hypoxia reduces the sensitivity of EMT6/Ro cells to doxorubicin (Adriamycin), 5-fluourouracil, and actinomycin D because of an amplification of the P-glycoprotein gene family (Sakata et al., 1991). Induction of P-glycoprotein as well as BCRP and the organic anion-transporting polypeptide 2 (OATP2) frequently occurs through activation of PXR (Anapolsky et al., 2006); however, this mechanism did not appear to play an important role in hypoxic animals because mRNA levels of BCRP and OATP2 as well as PXR were not affected. Alternatively it is plausible that binding sites for HIF-1, found on the promoter region of MDR1 (Krishnamurthy et al., 2004), may be involved in the observed hypoxia-mediated induction of P-glycoprotein. Hypoxia-induced resistance to chemotherapy, which is associated with the overexpression of Mdr1 and MRPI genes, is believed to be mediated through HIF-1 activation, depending in part upon signaling via the stress-activated protein kinase c-Jun NH2-terminal kinase (Comerford et al., 2004). Recent reports also indicate that HIF-1α silencing increases chemosensitivity of non-small cell lung cancer and breast carcinoma to cisplatin, doxorubicin, and methotrexate (Song et al., 2006). Overall, these studies suggest that multiple signaling pathways may be implicated in hypoxia-induced drug resistance (Dixit et al., 2005).

Besides systemic hypoxia, there are other experimental pathologic conditions that can modulate the expression of membrane transporters. For instance, acute renal and acute hepatic failure (Murakami et al., 2002), as well as local and systemic inflammatory reactions (Ho and Piquette-Miller, 2006), reduces the expression of P-glycoprotein.

**TABLE 1**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CYP1A1</th>
<th>CYP1A2</th>
<th>CYP2B4</th>
<th>CYP2C5</th>
<th>CYP2C16</th>
<th>CYP3A6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-IL-1β</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-IL-2</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Anti-IL-6</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Anti-INF-γ</td>
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<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Anti-Epo</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Effect of cytokines on the expression of CYP1A1, 1A2, 2B4, 2C5, 2C16, and 3A6**

Hepatocytes were incubated with serum from rabbits subjected to 48 h of hypoxia in the presence of an anti-cytokine antibody for 24 h. The + signs indicate the effect of immunoneutralization on the expression of the isoform; − indicates no effect on the isoform.
The fact that in vivo acute moderate systemic hypoxia increases the expression of P-glycoprotein may have clinical implications concerning drug response in patients presenting acute hypoxia of respiratory or cardiac origin with or without nocturnal desaturation.

In conclusion, acute moderate hypoxia diminishes the expression of CYP1A1, 1A2, 2B4, 2C5, and 2C16, an effect mediated by IFN-γ, IL-1β, and IL-2, but increases that of CYP3A4, an effect partially mediated by Epo. In addition, acute moderate hypoxia increases the expression of P-glycoprotein. The net result of these changes on the kinetics of drugs will depend upon the isoform involved in their biotransformation. We may predict that the area under the concentration-time curve of substrates of CYP3A will be smaller because of biotransformation. We may predict that the area under the concentration-time curve of substrates of CYP3A will be smaller because of biotransformation.

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References


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