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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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Running title: Hypoxia and cytochrome P450 and P-glycoprotein

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Abbreviations: AP-1 is activator protein-1; AhR is aryl hydrocarbon receptor; Arnt is aryl hydrocarbon receptor nuclear translocator; CAR is constitutive androstane receptor; Epo is erythropoietin; Erk is extracellular signal-regulated kinase; FiO_2 is fractional concentration of inspired O_2; H_{CONT} is hepatocytes from control rabbits; H_{HYPO} is hepatocytes from rabbits with hypoxia; HIF-1 is hypoxia-inducible factor 1 transcriptional activator; IFN-γ is interferon-γ; IL-1β is interleukin-1β; MDR1 is multidrug resistance protein 1; M_r is relative molecular mass; OATP2 is organic anion transporting polypeptide 2; P450 is cytochrome P450; PXR is pregnane X receptor; ROS is reactive oxygen intermediates; S_{CONT} is serum from control rabbits; S_{HYPO} is serum from rabbits with hypoxia; TNF-α is tumour necrosis factor; WME is William's medium E.

Abstract

In vivo, 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, since 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 O_2 of 34 ± 1 mmHg. Compared with rabbits breathing room air, hypoxia 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 neutralisation experiments. The serum mediators responsible for the down-regulation of P450 isoforms were interferon-γ, interleukin-1β (IL-1β) and IL-2. *In vivo*, in 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 upregulated in the livers of hypoxic rats.

Introduction

In patients with cardio-respiratory diseases, acute hypoxemia appears to reduce the ability of cytochrome P450 isoforms to catalyze the biotransformation of xenobiotics. Patients with pulmonary insufficiency showing 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, while CYP2D6, 2E1 and 3A4 exhibit low affinity and variable capacity (Ha et al., 1995). Rabbits exposed to a fractional concentration of inspired O_2 (FiO_2) 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), interleukine-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 (CAR) (Fradette and du Souich, 2003).

There is indirect evidence that in addition to CYP1A1 and 1A2, hypoxemia down-regulates other cytochrome P450 isoforms. Effectively, antipyrine half-life was increased 120% in a group of patients predominantly with chronic hypoxemia (PaO_2 < 55mm Hg) from a variety of causes,

when compared with other subjects without severe hypoxia (Cumming, 1976). In humans, antipyrine is metabolized by numerous hepatic CYP isozymes, e.g. CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19 and 3A4 (Engel et al., 1996; Sharer and Wrighton, 1996).

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 isoforms. On the other hand, P-glycoprotein is modulated by CAR (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.

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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, Québec, 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 x 1.20 x 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) which utilizes nitrogen to displace oxygen from the chamber. This FiO_2 generates a stable arterial partial pressure of O_2 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 (FiO_2 = 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 (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 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 KH_2PO_4, 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, $CaCl_2$ (1 mM), and trypsin inhibitor (0.25 mM). 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 x 10^6 cells per ml 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) coated with type 1 rat tail collagen and incubated for 2 h at 37°C in an atmosphere of 95% air and 5% CO_2 to allow for 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 (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.

Proteins in the serum were separated by size exclusion high performance liquid chromatography (HPLC) on a Superose 12 HR column from Pharmacia Biotech (Baie d'Urfé, Québec, Canada) as previously described (Fradette et al., 2002). Briefly, flow rate of the mobile phase was set at 0.3 ml min^{-1} and column pressure was maintained between 9 to 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 115 nM NaCl, 5 nM KCL, 1 nM KH_2PO_4, 1 nM HEPES, 25 nM EGTA, and 5.5 nM glucose; the pH of the solution was adjusted to 7.4 and filtered through a 0.22 µm membrane. Serum aliquots

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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 fractions containing proteins with M_r between 9 and 14 kDa, 15 and 23 kDa, 24 and 31 kDa, 32 and 44 kDa, 45 and 64 kDa, and 65 and 94 kDa were separated and collected. Based on the size exclusion range of the column, proteins isolated in the first fraction were assumed to have a M_r greater than 95 kDa. In order to increase the inhibitory activity of the fractions collected, these were concentrated on Microsep 3K membranes (Pall Filtron, Northborough, MA. U.S.A.) that retain proteins greater than 3 kDa. Three ml of the fractions were added to the sample reservoir and centrifuged at 75000 x g to reduce the volume and hence concentrate serum fractions 1.25 times.

The medium of the cultured hepatocytes was replaced with fresh WME supplemented with 10% calf serum and 1 µM insulin, and hepatocytes (H_{CONT} and H_{HYPO}) were then incubated with 200 µl of serum from control rabbits (S_{CONT}), 200 µl of serum from hypoxic rabbits (S_{HYPO}), 200 µl of the HPLC fractions of S_{CONT} and S_{HYPO}, or 200 µl of saline, e.g. in absence of serum or its fractions, for 24 h. Following 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 immuno-neutralization. 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 isoforms expression, and b) 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-IL2), 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 inter-species reactivity of these antibodies (Fradette et al., 2002). An IgG monoclonal antibody to *Pseudomona 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 since this amount is effective to immuno-neutralize 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 PBS and centrifuged at 1500g for 5 min. The pellet was resuspended in cold lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 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 (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 nitro-cellulose 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% non-fat milk in TBS-T (0.1% Tween 20) and visualized with an

alkaline phosphatase conjugated secondary antibody using nitro blue tetrazolium as substrate. CYP2B4, 2C5, 2C16 and CYP3A6 proteins were detected with rabbit anti-human antibodies, that recognize rabbit isoforms, against CYP2B4 diluted 1:500 (Research diagnostics, Flanders, NJ, U.S.A.), CYP2C5 diluted 1:100 (Research diagnostics, Flanders, NJ, U.S.A.), CYP2C16 diluted 1:100 (Research diagnostics, Flanders, NJ, U.S.A.) and CYP3A6 diluted 1:500 (Oxford Biochemical Research, Oxford, MI, U.S.A.), 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. 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 due to the lack of information and commercial availability of antibodies for transporters in rabbit. Preliminary experiments showed that a FiO_2 of 8% reduced the arterial partial pressure to similar levels as those measured in rabbits, e.g. 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 FiO_2 of 8% in the chamber as described above. Control rats were placed into chambers which contained room air (FiO_2 = 21%) for the experiments. Forty eight hours later, the rats were anaesthetized with halothane and the liver removed, rapidly frozen in liquid N_2 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 (Amersham Biosciences, Piscataway, NJ) according to manufacturer's protocol, and cDNA was synthesized from 2.5 μg RNA using the First Strand cDNA Synthesis kit (MBI Fermentas, Flamborough, ON). Transporter mRNA levels were measured by real-time quantitative PCR 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 (Kalitsky-Szirtes et al., 2004). Primers for BCRP were fwd 5'-AGT CCG GAA AAC AGC TGA GA-3' and rev 5'-CCC ATC ACA ACG TCA TCT TG-3'; and for OATP2 were fwd 5'-TTG GTG TTG GAT GTG CAG TT-3' and rev 5'-GCC AAT GGT CAT TCC TGT TT-3'. All mRNA levels were normalized to 18S mRNA and the ratios presented as % control.

The hepatic crude membrane fraction was isolated as described previously (Teng and Piquette-Miller, 2005) and measured by the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) (Bradford, 1976). For the immunoblotting, 10 μg of protein was loaded and run on a 10% acrylamide gel and transferred onto a Hybond ECL nitrocellulose membrane (Amersham Biosciences Inc.). Membranes containing transporter protein were then cut in half and the upper portion (mol. wt. >78 kDa), was incubated overnight with 2 μg of the P-

glycoprotein monoclonal antibody, C219 (ID 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 kDa) was incubated with anti-β-actin clone AC15 antibody (Sigma-Aldrich) followed by sheep anti-mouse IgG (Amersham). Bands were detected using an ECL Western blotting analysis system (Amersham), imaged on Bioflex MSI film (Clonex Corp., InterSciences Inc., Markham, ON, Canada), and quantified using Kodak Digital Science 1D Image Analysis software (Eastman Kodak, Rochester, NY).

Drugs and Chemicals

Percoll gradient, William's medium E, calf serum, type I rat tail collagen, NaCl, KCl, KH_2PO_4, Hepes, EGTA, glucose, were purchased from Sigma Chemicals (Sigma, St. Louis, MO, U.S.A.). 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-CYP2B4, anti-CYP2C5 and anti-CYP2C16 antibodies from Research diagnostics (Flanders, NJ, 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.).

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 (ANOVA), followed by the Newman-Keuls *post hoc* test. Differences were considered significant when $p<0.05$.

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Results

Effect of hypoxia *in vivo* on hepatic P450 isoform expression

Exposure of rabbits to an 8 % FiO_2 atmosphere for 48 h generated a stable hypoxemia, with an average arterial partial pressure of O_2 (PaO_2) of 34 ± 1 mmHg, without influencing the arterial partial pressure of CO_2 (≈ 21 ± 1) and arterial pH (7.47 ± 0.05). Compared with rabbits breathing a 21% FiO_2 (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% (Figure 1).

Effect of serum from control rabbits (S_{CONT}) and serum of rabbits with hypoxia (S_{HYPO}) on hepatic P450 isoform expression in H_{CONT} and in H_{HYPO}

As compared to H_{CONT} incubated with S_{CONT}, incubation of H_{CONT} with S_{HYPO} for 24 h reduced the amount of CYP2C5 and 2C16 by 15% and 17%, respectively (p<0.05). Moreover, S_{HYPO} increased the amount of CYP3A6 by 31% (p<0.05, n = 6). No changes in CYP1A1, 1A2 and 2B4 proteins were observed (Figure 2A).

In H_{HYPO} incubated with S_{CONT}, the expression of CYP1A1, 1A2, 2B4, 2C5 and 2C16 were reduced by 33, 35, 42, 70, and 77%, respectively (p<0.05, n = 6), and that of CYP3A6 was increased by around 70% (p<0.05), compared with H_{CONT} (Figure 2B). Following 24 h incubation of H_{HYPO} with S_{HYPO}, the amount of CYP1A1 and 1A2 proteins were 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 H_{HYPO} with S_{HYPO} increased the expression of CYP3A6 by an average of 50% (p<0.05) (Figure 2B).

Effect of S_{HYPO} HPLC fractions on P450 isoform expression in H_{HYPO}

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As compared with the S_{CONT}, incubation of the S_{HYPO} 15-23 kDa HPLC fraction with H_{HYPO} for 24 h, decreased expression of CYP1A1, 1A2 and CYP3A6 proteins by 42, 30% and 21%, respectively ($p<0.05$, n = 6), whereas CYP2B4, 2C5 and 2C16 were reduced to undetectable levels (Figure 3). On the other hand, incubation of H_{HYPO} with the S_{HYPO} 32-44 kDa fraction increased the expression of CYP3A6 protein by 52% ($p<0.05$, n = 7) (Figure 3). The remaining S_{HYPO} HPLC fractions did not affect the expression of any of the P450 isoforms studied compared with S_{CONT}. Likewise, 24 h incubations of S_{CONT} fractions with H_{CONT} did not modify the expression of CYP1A1, 1A2, 2B4, 2C5, 2C16 and 3A6 (data not shown).

Identification of the mediators in S_{HYPO} responsible for the changes in the expression of P450 isoforms

As compared with the incubation with the S_{HYPO} 15-23 kDa fraction, which significantly reduced the expression of all CYP isoforms in H_{HYPO} cells, co-incubation 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, 2B4, 2C5 and 2C16 (n = 6); 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).

While the S_{HYPO} 32-44 kDa fraction significantly increased CYP3A6 expression by around 80%; co-incubation of an anti-Epo antibody (n = 6) to the 32-44 kDa fraction attenuated CYP3A6 protein induction by 40% ($p<0.05$). However, CYP3A6 expression was still greater ($p<0.05$) than that observed in the S_{CONT} treated H_{HYPO} cells (Figure 4).

Effect of moderate hypoxia on the expression of membrane transporters

As compared with control rats (n = 6), the expression of CYP3A1 increased by 75% ($p<0.05$, n = 9) in hypoxic rats exposed to a FiO_2 of 8% for 48 h (Figure 5). Levels of CYP3A1 mRNA were also similarly induced in hypoxic rats (data not shown). Levels of *mdr1b* mRNA was significantly induced in hypoxic rats($p<0.05$, n = 9), however hypoxia did not affect the expression of *Mdr1a*, *OATP2* and *BCRP* mRNAs. Likewise, protein levels of P-glycoprotein were significantly increased by 77% in hypoxic rats ($p<0.05$, n = 9) (Figure 7). Hypoxia tended to increase *PXR* mRNA ($p>0.05$, n = 9) (Figure 6).

Discussion

The present study demonstrates that besides CYP1A1 and 1A2, acute moderate hypoxia down-regulates CYP2B4, 2C5, and 2C16 *in vivo*, the orthologs of human CYP2B6, 2C9 and 2C19, respectively. The serum mediators contributing to the decrease in expression of these isoforms are IFN-γ, IL-1β and IL-2. This study confirms that acute moderate hypoxia as well as serum from rabbits with hypoxemia up-regulate the expression of CYP3A6, in part due to Epo (Fradette et al., 2002). Hypoxia *in vivo* and S_{HYPO} induce the expression of CYP3A6 through a protein tyrosine kinase- and an extracellular signal-regulated kinase 1/2 (Erk1/2)-dependent activation of HIF-1 and AP-1 nuclear translocation (Fradette and du Souich, 2003). The decrease in CYP1A1 and 1A2 expression by hypoxia occurs at the transcriptional level (Fradette et al., 2002).

The serum mediators responsible for the down-regulation of CYP1A1, 1A2, 2B4, 2C5 and 2C16 are primarily IFN-γ and IL-1β, with IL-2 playing a minor role (Table 1). It is noteworthy that 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 turpentine-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, e.g. 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 fade when incubations are conducted for longer periods, e.g. 48 h (El-Kadi et al., 1997; Bleau et al., 2003). The differences in response to serum may be associated to 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 (ROS) 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 which 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 enzymes. These target genes are induced by a common transcription factor, HIF-1 (Aragones et

al., 2001). Hypoxia, cytokines and ROI stabilize hypoxia-inducible factor-1α (HIF-1α) which translocates to the nucleus where it dimerizes with HIF-1β or aryl hydrocarbon receptor nuclear translocator (Arnt) to form HIF-1. Since 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 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).

Activator protein-1 (AP-1) is activated by hypoxia by the increase in intracellular calcium. Hypoxia, increases intracellular calcium that activates Erk1/2 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 (NRE) 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, TNF-α and IFN-γ (Naldini et al., 1997). The increase in IL-1β, IL-2, and IFN-γ activate transcription factors, such as NF-κB, AP-1 and c-myc (Wesselborg 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, probably multiple mechanisms 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 is not affected by hypoxia, two drugs primarily metabolized by CYP3A6 in rabbits (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-fluorouracil, and actinomycin D because of an amplification of P-glycoprotein gene family (Sakata et al., 1991). Induction of P-glycoprotein as well as the breast cancer resistance protein (BCRP) and the organic anion transporting polypeptide 2 (OATP2) frequently occur through activation of the pregnane X receptor (PXR) (Anapolsky et al., 2006), however this mechanism did not appear to play an important role in hypoxic animals as 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

MRP1 genes, is believed to be mediated through HIF-1 activation, depending in part through signaling via the stress-activated protein kinase c-Jun NH_2-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 pathological 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) reduce the expression of P-glycoprotein. 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, effect mediated by IFN-γ, IL-1β and IL-2, but increases that of CYP3A6, 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 (AUC) of substrates of CYP3A will be smaller do to decreased absorption, and increased first pass and systemic/renal clearance. For substrates of the sub-families CYP1A, 2B and 2C, hypoxia shall reduce their clearance and the repercussions on the AUC will depend on whether these drugs are or not substrates of P-glycoprotein. On the other

hand, by enhancing its expression, the pharmacologic response elicited by substrates of P-glycoprotein may be reduced.

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Footnotes

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Legends for figures

Figure 1. Effect of hypoxia on the expression of cytochrome P450 isoforms. Rabbits were exposed *in vivo* for 48 h to a 8% FiO_2 to produce an acute moderate hypoxia and the amount of CYP1A1, 1A2, 2B4, 2C5, 2C16 and 3A6 apoproteins was measured in hepatocytes by immunoblot. H_{CONT} (n = 6) and H_{HYPO} (n = 6) are hepatocytes harvested from control rabbits and rabbits with acute moderate hypoxia. The upper panel contains representative blots from control and rabbits with hypoxia. Data is mean ± standard error of densitometric ratios. * $p<0.05$ compared with control.

Figure 2. Effect of serum from rabbits on the expression of cytochrome P450 isoforms. Serum from control rabbits (S_{CONT}) and serum from rabbits with acute moderate hypoxia (S_{HYPO}) were incubated for 24 h with hepatocytes from control rabbits (H_{CONT}, Panel A, n = 6) and from rabbits with acute moderate hypoxia (H_{HYPO}, Panel B, n = 6) and the amount of CYP1A1, 1A2, 2B4, 2C5, 2C16 and 3A6 apoproteins was measured in hepatocytes by immunoblot. Data is mean ± standard error of densitometric ratios. * $p<0.05$ compared with S_{CONT}; ** $p<0.05$ compared with S_{CONT} incubated with H_{CONT}.

Figure 3. Effect of serum and its HPLC fractions on the expression of cytochrome P450 isoforms. Serum from control rabbits (S_{CONT}), serum from rabbits with acute moderate hypoxia (S_{HYPO}), and the HPLC serum fractions of 23-15 kDa and 44-32 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 is mean ± standard error of densitometric ratios. * $p<0.05$ compared with S_{CONT}.

Figure 4. Serum mediators implicated in the changes in cytochrome P450 isoforms induced by hypoxia. Effect of anti-cytokine antibodies on the ability of the 44-32 kDa and 23-15 kDa serum

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fractions to modify the expression CYP1A1, 1A2, 2B4, 2C5, 2C16 and 3A6 apoproteins incubated for 24 h with hepatocytes from rabbits with acute moderate hypoxia (n = 6). S_{CONT} is serum from control rabbits; Epo is erythropoietine; IL-2, IL-1β, IL-6 and IFN-γ are interleukin-2, -1β, -6 and interferon-γ, respectively. Data is mean ± standard error of densitometric ratios (* $p<0.05$ compared with control).

Figure 5. Effect of hypoxia on CYP3A1 expression in rat liver. Rats were exposed to room air (control) or to a FiO_2 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 in methods. Data is represented 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.

Figure 6. Effect of hypoxia on transporter and PXR mRNA expression in rat liver. Rats were exposed to room air (control) or to a FiO_2 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 PCR and normalized to levels of 18S mRNA as described in methods. Bars graphs represent the mean± standard error in control (n=6) and hypoxic (n=9) rats (*$p<0.05$ versus controls).

Figure 7. Effect of hypoxia on P-glycoprotein protein expression in rat liver. Rats were exposed to room air (control) (n = 6) or to a FiO_2 of 8% (n = 9) for 48 h. The liver was harvested, and the crude membrane protein fraction was isolated. P-gp levels were measured by western blotting as described in Materials and Methods, and normalized to β-actin levels. Vertical bars are SEM. *$p<0.05$ versus controls.

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Table 1. 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 hypoxia in the presence of an anti-cytokine antibody for 24 h. The signs + and – indicate the effect of the immuno-neutralization on the expression of the isoforms; ≈ indicates no effect on the isoform.

	CYP					
Antibody	1A1	1A2	2B4	2C5	2C16	3A6
Anti-IL-1β	+	≈	+	+	+	≈
Anti-IL-2	+	≈	≈	≈	+	≈
Anti-IL-6	≈	≈	≈	≈	≈	≈
Anti-INF-γ	++	++	+++	++	++	+
Anti-Epo	≈	≈	≈	≈	≈	+

Figure 1

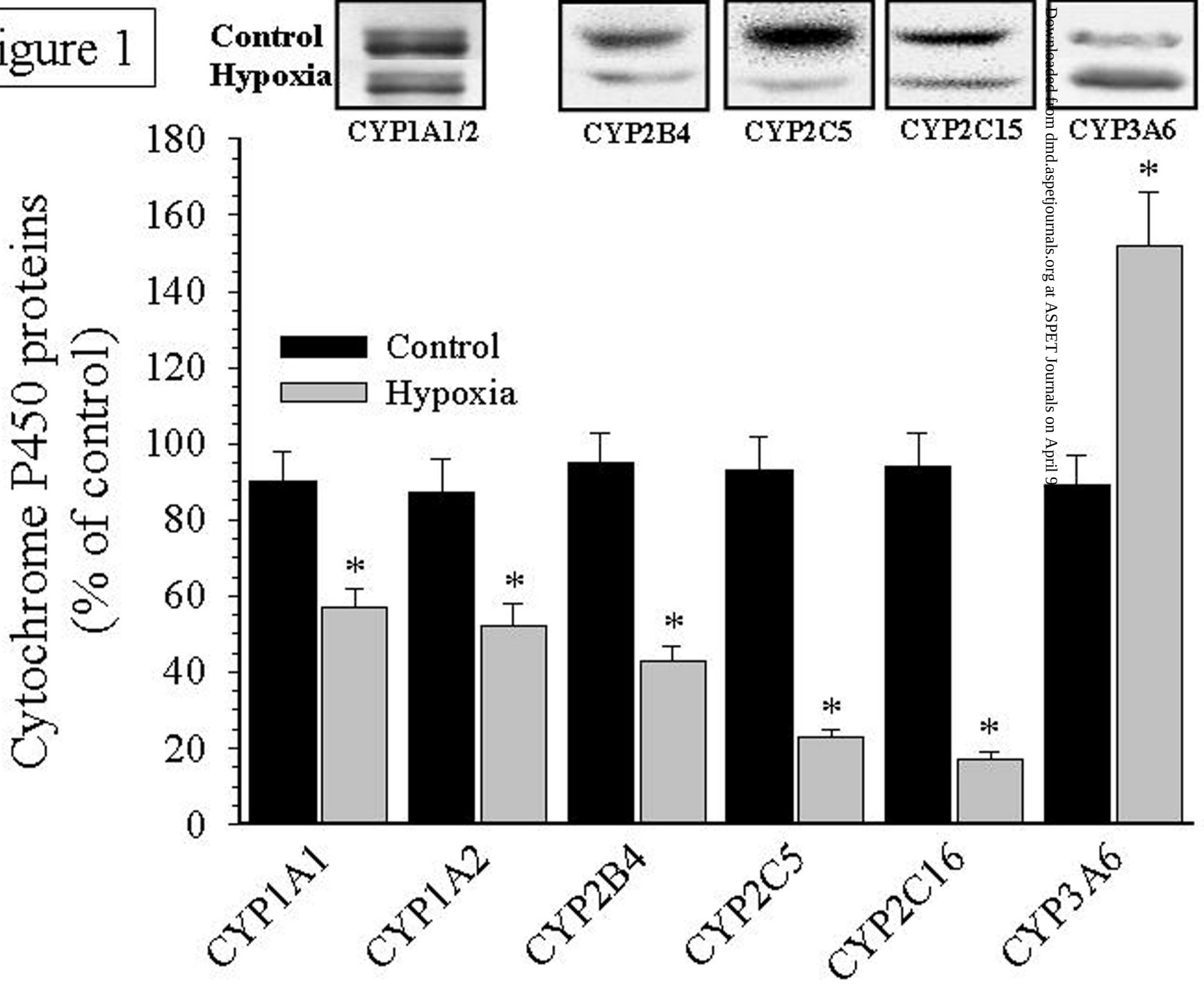

Figure 2

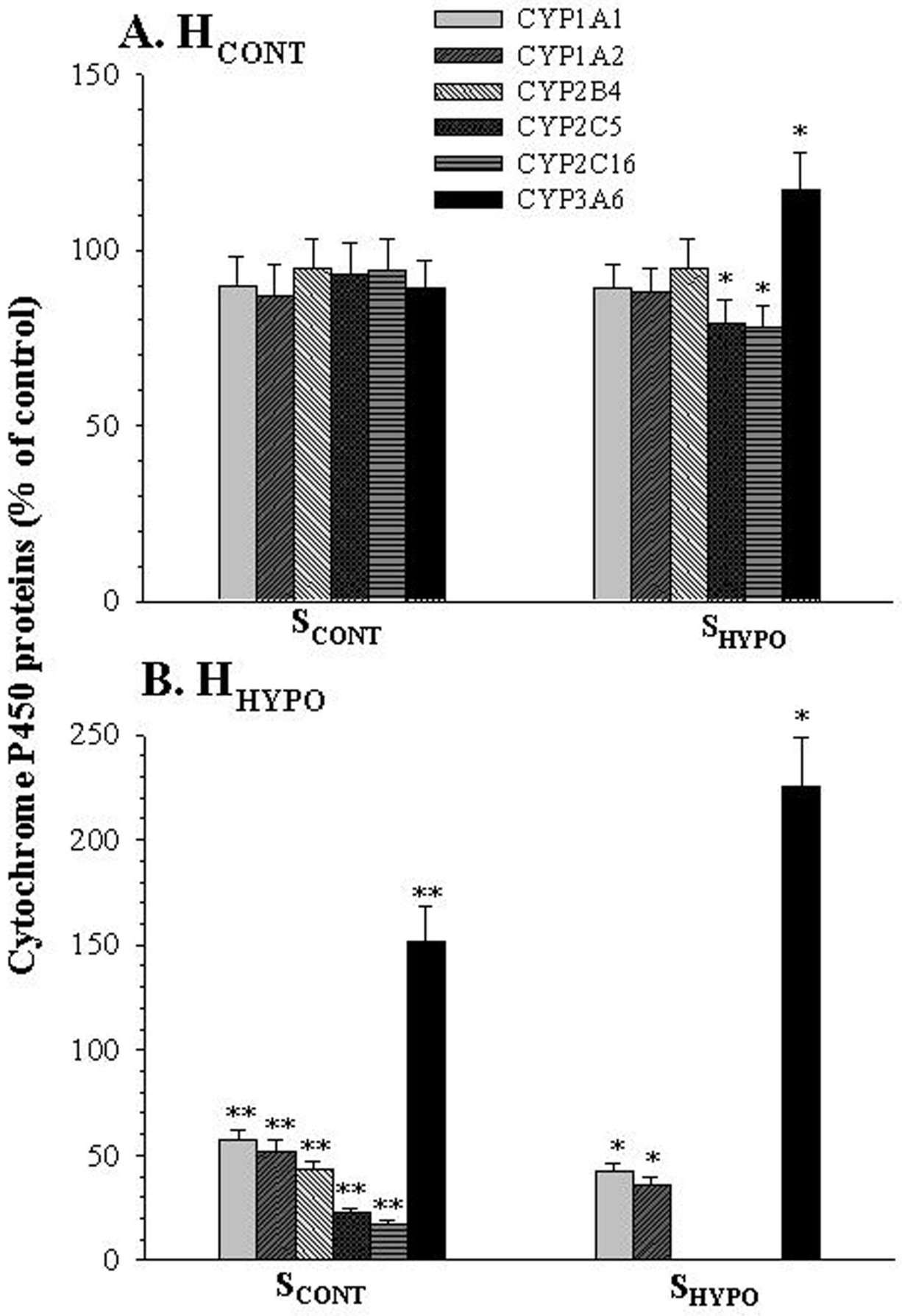
A. H CONT
CYP1A1
CYP1A2
CYP2B4
CYP2C5
CYP2C16
CYP3A6
150
100
50
0
Cytochrome P450 proteins (% of control)
S CONT
S HYPO
B. H HYPO
250
200
150
100
50
0
S CONT
S HYPO

Figure 3

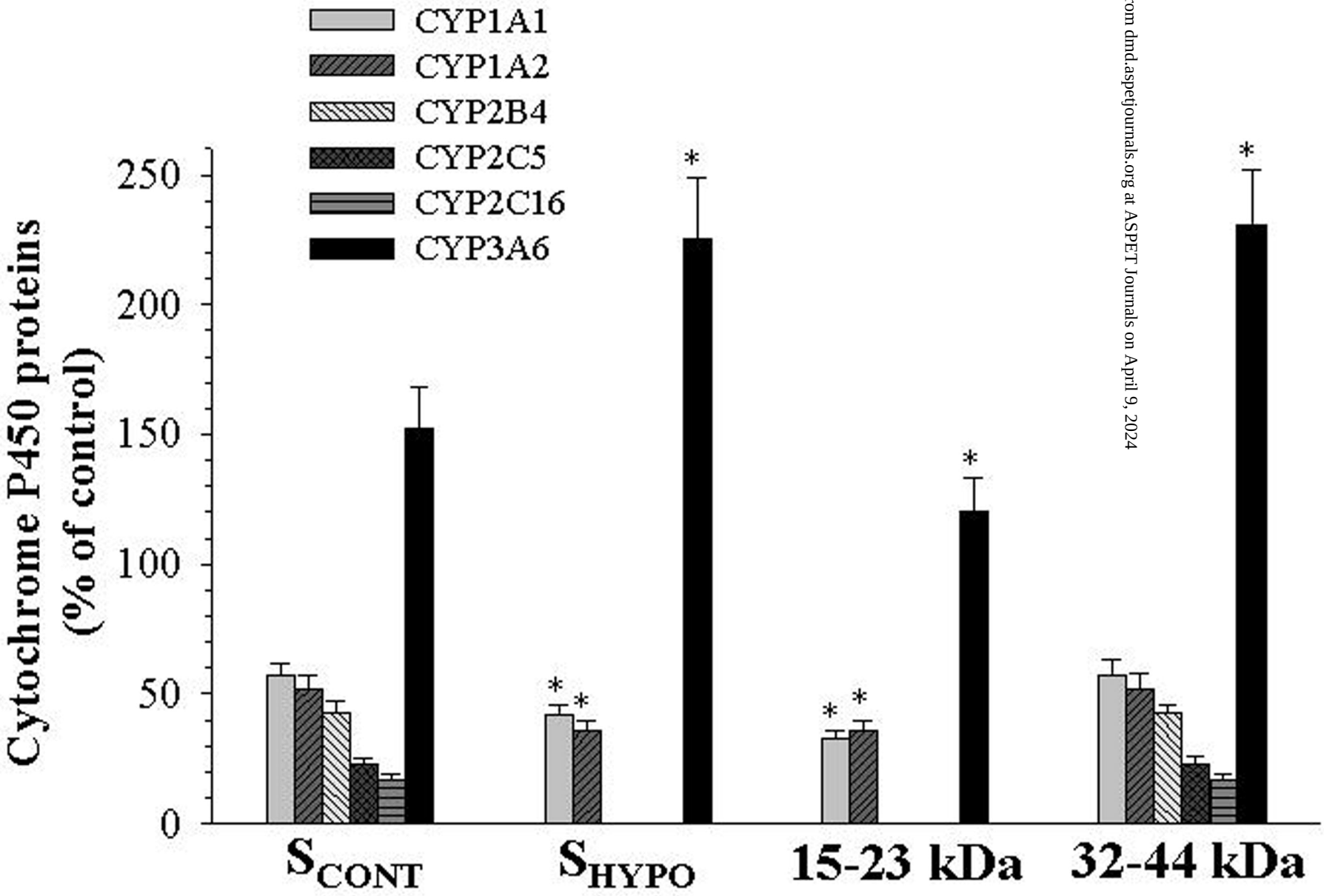

Figure 4

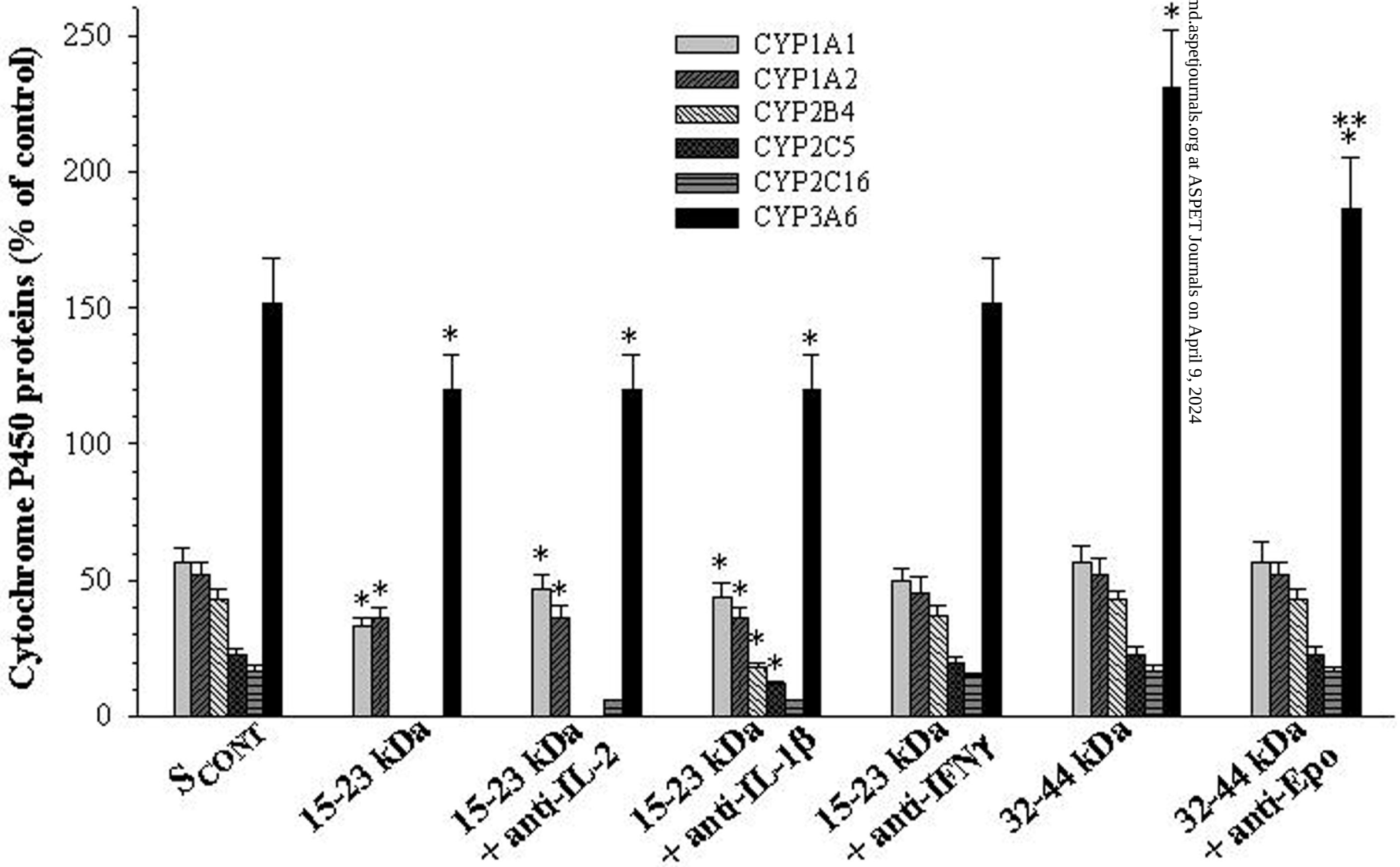

Figure 5

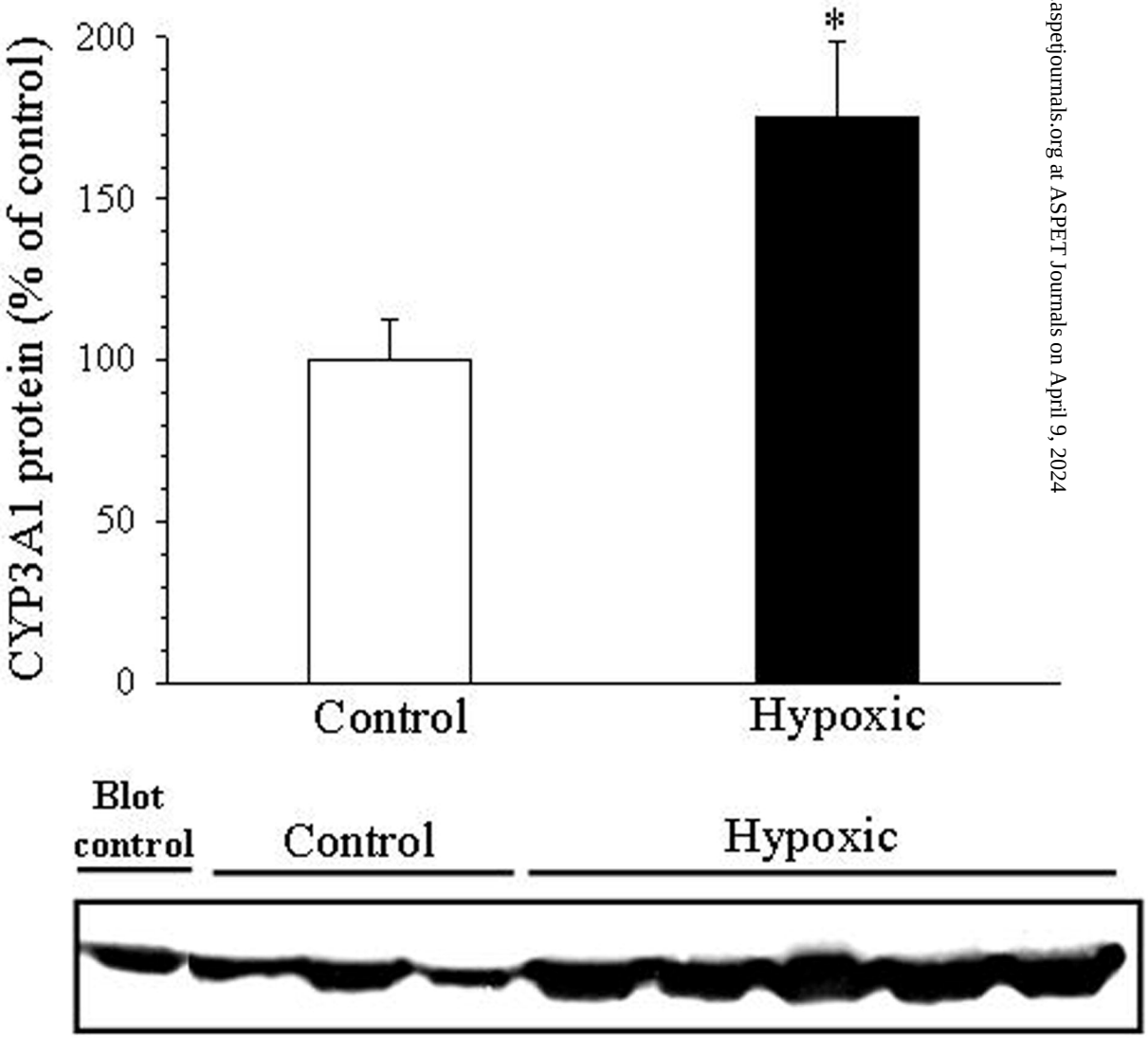

Figure 6

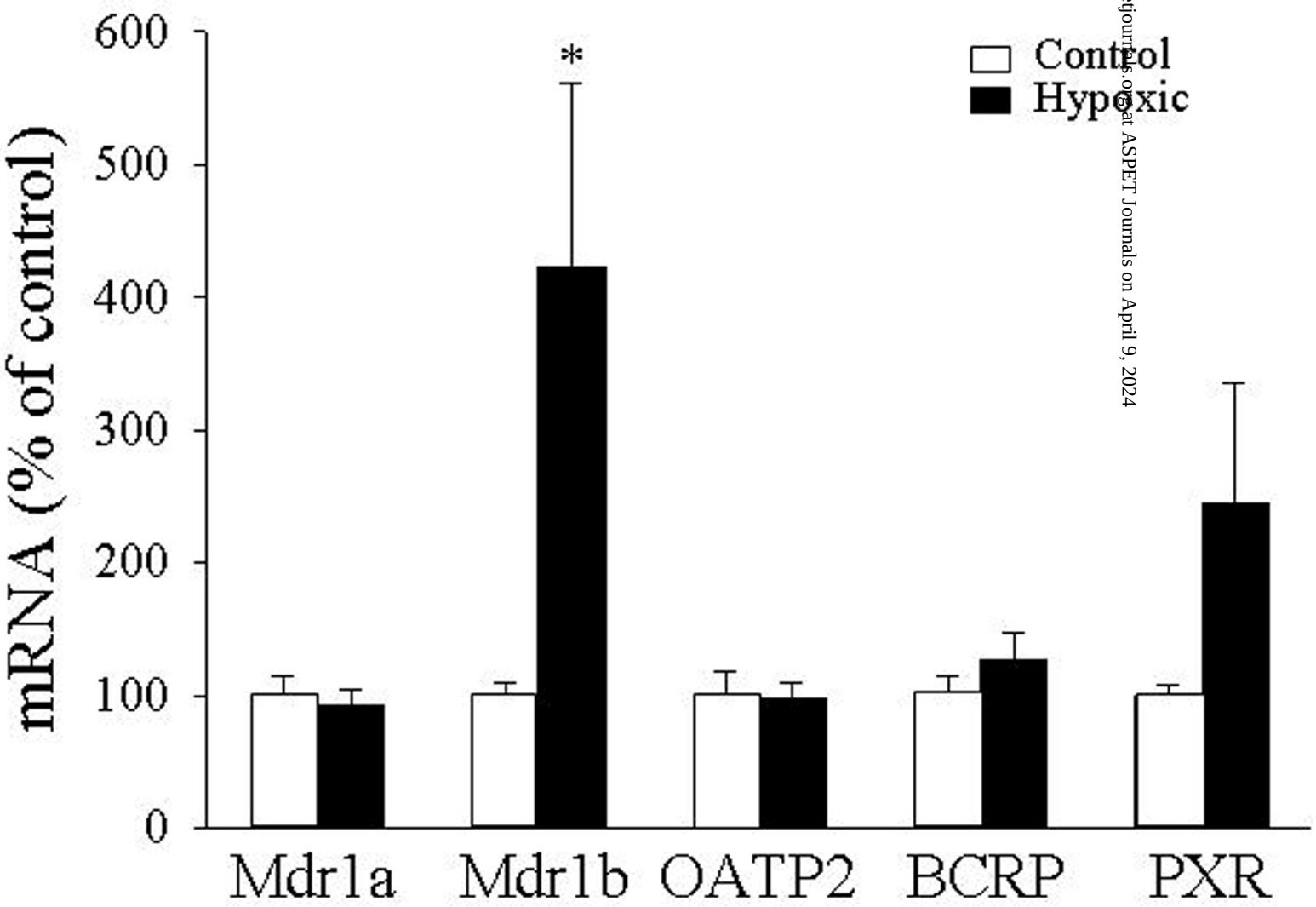

Figure 7