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Effect of hypoxia alone or combined with inflammation and 3-methylcholanthrene on hepatic cytochrome P450 in conscious rabbits

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1 To investigate the effect of moderate hypoxia alone or combined with an inflammatory reaction or after 3-methylcholanthrene (3MC) pre-treatment on cytochrome P450 (P450), conscious rabbits were exposed for 24 h to a fractional concentration of inspired O_2 of 10% (mean PaO_2 of 34 mmHg). Hypoxia decreased theophylline metabolic clearance (Cl_M) from 1.73 ± 0.43 to 1.48 ± 0.13 ml min^{-1} kg^{-1} ($P<0.05$), and reduced ($P<0.05$) the formation clearance of theophylline metabolites, 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU). Hypoxia reduced the amount of CYP1A1 and 1A2 but increased CYP3A6 proteins.

2 Turpentine-induced inflammatory reaction reduced ($P<0.05$) the formation clearance of 3MX, 1MU, and 1,3DMU, and diminished the amount of CYP1A1, 1A2 and 3A6 proteins. However, when combined with hypoxia, inflammation partially prevented the decrease in Cl_M, especially by impeding the reduction of 1,3DMU. The amount of CYP1A1 and 1A2 remained reduced but the amount of CYP3A6 protein returned to normal values.

3 Pre-treatment with 3MC augmented the Cl_M by 114% ($P<0.05$) due to the increase in the formation clearance of 3MX, 1MU and 1,3DMU. 3MC treatment increased the amount of CYP1A1 and 1A2 proteins. Pre-treatment with 3MC prevented the hypoxia-induced decrease in amount and activity of the P450.

4 It is concluded that acute moderate hypoxia and an inflammatory reaction individually reduce the amount and activity of selected apoproteins of the P450. However, the combination of hypoxia and the inflammatory reaction restores P450 activity to near normal values. On the other hand, pre-treatment with 3MC prevents the hypoxia-induced depression of the P450.

Keywords: Hypoxia, inflammation; 3-methylcholanthrene; cytochrome P450; theophylline; kinetics; conscious rabbits

Abbreviations: $AUC_{0\rightarrow t}$, area under theophylline plasma concentration-time curve from 0 to t; $AUC_{0\rightarrow\infty}$, area under theophylline plasma concentration-time curve from 0 to ∞; Cl_M, theophylline metabolic clearance; Cl_{M3MX}, fraction of theophylline metabolic clearance yielding 3MX; Cl_R, theophylline renal clearance; Cl_S, theophylline systemic clearance; COLD, chronic obstructive lung disease; 1,3DMU, 1,3-dimethyluric acid; FiO_2, fractional concentration of inspired O_2; $F_{3MX}M$, fraction of theophylline being metabolized to 3MX; F_IM, fraction of theophylline undergoing metabolism; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; 3MC, 3-methylcholanthrene; 1MU, 1-methyluric acid; 3MX, 3-methylxanthine; $NO^{\bullet}$, nitric oxide; $O_2^{\bullet -}$, superoxide anion; P450, cytochrome P450; $PaCO_2$, arterial partial pressure of CO_2; PaO_2, arterial partial pressure of O_2; ROI, reactive oxygen intermediates; Vd_{SS}, predicted theophylline volume of distribution at steady state; X_{U3MX}, amount of 3MX recovered in urine; z, terminal rate constant of disposition

Introduction

Compared with healthy subjects and patients with uncomplicated asthma, in patients with chronic obstructive lung disease (COLD), pulmonary oedema, pulmonary heart disease, or congestive heart failure, the clearance of theophylline can be reduced by as much as 30–60% (Hendeles *et al*., 1977; Powell *et al*., 1978). Since patients with these disease states present hypoxia, the decrease in theophylline rate of metabolism has been associated to hypoxia (Jacobs & Senior, 1974). This assumption is supported by *in vivo* animal studies showing that moderate hypoxia decreases the clearance of theophylline (Letarte & du Souich, 1984).

In man, an inflammatory reaction such as an acute viral respiratory infection enhances theophylline plasma concentrations secondary to a decrease in its clearance (Chang *et al*., 1978); bacterial pneumonia reduces the clearance of theophylline and of antipyrine (Vozeh *et al*., 1978; Sonne *et al*., 1985), and BCG vaccination is able to decrease theophylline clearance (Gray *et al*., 1983). In animal models, non-infectious inflammatory reactions, such as those induced by the injection of turpentine, carrageenan or Freund's adjuvant, also diminish the rate of biotransformation of xenobiotics (Beck & Whitehouse, 1974; Parent *et al*., 1992). Although it is accepted that hypoxia and an inflammatory reaction individually depress the activity of the cytochrome P450 (P450) (Richer & Lam, 1993), in patients with advanced COLD, the effect of hypoxia on the P450 remains controversial (Cusack *et al*., 1986; du Souich *et al*., 1989), perhaps because the combined effect of hypoxia and an inflammatory reaction, as seen in patients with advanced COLD, on the P450 may not be as predicted from each individual condition.

The aims of the present study were to document *in vivo* the effect of acute moderate hypoxia alone and combined with an inflammatory reaction, a condition that down-regulates the

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P450, on hepatic P450, or combined with 3-methylcholanthrene pre-treatment (3MC), a condition that induces selective apoproteins of the P450. The activity of the P450 was assessed by administering theophylline to conscious rabbits and measuring the rate of formation of its metabolites. Theophylline was used as a substrate because its major metabolite, 1,3DMU, results from theophylline 8-hydroxylation catalyzed in man by several P450 apoproteins, such as CYP1A2, 2D6, 2E1, and 3A4. Minor metabolites include 3MX result of a 1-demethylation carried out by CYP1A2, and 1-methylxanthine product of a 3-demethylation catalyzed by CYP1A1 and CYP1A2, which is 8-hydroxylated by xanthine oxidase to yield 1MU (Sarkar & Jackson, 1994; Zhang & Kaminsky 1995). In addition, it was of interest to assess the repercussion of these experimental conditions on the amount of hepatic P450 apoproteins involved in the metabolism of theophylline, essentially CYP1A1 and 1A2, and marginally 3A6, but the latter being very important for the metabolism of numerous drugs.

Methods

Animals

Male New Zealand white rabbits (2.2–3.6 kg) purchased from Les Lapins Léonard Inc. (St-Vincent Mirabel, Québec, Canada) were used throughout the study. They were maintained on Purina pellets and water *ad libitum* for at least 1 week before any experimental work was undertaken. Before each experiment, a sterile Bardex Foley catheter (No. 8–10) was introduced into the bladder of the animals. To stabilize the physiologic parameters 1 h before the experiment started, the rabbits were placed into restraining cages (Plaslabs, Lansing, MI, U.S.A.), and a catheter (Butterfly 21, Abbott Ireland Ltd) was inserted into the central artery of an ear for blood sampling. A polyethylene tube (PE 50, Intramedic Becton, Dickinson and Company, Parsippany, NJ, U.S.A.) was introduced into a lateral vein of the ear for the infusion of a solution of 0.9% NaCl and 5% glucose (50/50) at a rate of 0.382 ml min^{-1} to replace basal losses through lungs and kidneys, and blood sampling. The rabbits were conscious throughout the experiment.

Experimental protocol

Effect of hypoxia on theophylline metabolism To induce the hypoxia, the rabbits were introduced in a plexiglas chamber ($0.75 \times 1.20 \times 1.25$ m^3), where a 10% fractional concentration of inspired O_2 (FiO_2) was 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. The 10% FiO_2 was selected to obtain an arterial partial pressure of O_2 (PaO_2) of approximately 35 mmHg. Humidity in the chamber was maintained at 50% by recirculating the air through a refrigerating system. The temperature was kept at 22–24°C. 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 ($FiO_2 = 21\%$). After 1 h of stabilization, the rabbits were left in the plexiglas chamber breathing room air ($n = 8$) or breathing an atmosphere with a low FiO_2 ($n = 8$) for 24 h, and then received 2.5 mg kg^{-1} of theophylline i.v. Blood samples (1.0 ml) were withdrawn at 0, 60, 90, 120, 125, 180, 240, 300, 360, 390, 420 and 480 min after the injection to assay theophylline in plasma. Urine was collected for 24 h while the rabbits were hypoxic in the chamber, to assay theophylline and its metabolites, e.g. 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU). Arterial blood samples (0.5 ml) were withdrawn at 0, 120, 360 and 450 min to monitor arterial blood gases and pH with a gas analyser (IL Micro 13-03/213-05, Instrumentation Laboratory, Lexington, MA, U.S.A.).

Effect of turpentine-induced inflammatory reaction on theophylline metabolism Control rabbits ($n = 8$) breathing air received 2.5 ml of a solution of sterile saline s.c. in both hind legs, and 48 h later, 2.5 mg kg^{-1} of theophylline i.v. Blood samples (1.0 ml) were withdrawn at 0, 5, 10, 15, 25, 35, 45, 60, 90, 120, 150, 180, 240, 300, 360, 420 and 480 min to assay theophylline. Urine was collected before and for 24 h after theophylline administration to assay theophylline, 3MX, 1MU and 1,3DMU. Additional blood samples (2.5 ml) were withdrawn prior to the administration of turpentine and 48 h later when theophylline was injected to assay seromucoids. Rectal temperature was measured with an electronic thermometer (Toshiba Digital Clinical Thermometer, Toshiba Glass Co. Ltd) prior to the injection of turpentine and 48 h after. After at least 10 days, the same eight rabbits received s.c. 2.5 ml of turpentine in both hind legs, to produce a chemical abscess with an inflammatory reaction (Ashton *et al*., 1970); 48 h later, they received 2.5 mg kg^{-1} of theophylline intravenously, and the protocol described above was applied. The turpentine-induced inflammatory reaction model was used because the serum of rabbits with a turpentine-induced local inflammatory reaction affects hepatic cytochrome P450 in a similar manner as do serum of humans with an upper respiratory viral infection (El-Kadi *et al*., 1997).

Combined effect of hypoxia and an inflammatory reaction on theophylline metabolism In preliminary studies, the lowest PaO_2 that rabbits with an inflammatory reaction could tolerate was determined to be 55 mmHg. The rabbits ($n = 6$) received turpentine as described above, and 24 h later, were introduced into the plexiglas chamber with a FiO_2 set at 12% and 24 h later, the rabbits received 2.5 mg kg^{-1} of theophylline i.v. To assay theophylline and seromucoids, blood samples and urine were collected as described earlier. To monitor arterial blood gases and pH (IL-Micro 13-03 pH/Blood Gas Analyser; Instrumentation Laboratory, Lexington, MA, U.S.A.), serial blood samples (0.25 ml) were withdrawn prior to and 120, 360 and 450 min after the administration of theophylline.

Combined effect of 3MC and hypoxia on theophylline metabolism Rabbits were segregated into four groups (7/group): the rabbits of the first and second groups received saline or corn oil s.c., rabbits of the third group received 80 mg kg^{-1} of 3MC suspended in corn oil s.c., and the rabbits of the fourth group received 80 mg kg^{-1} of 3MC suspended in corn oil s.c. and 24 h later were subjected to a 10% FiO_2. All the rabbits received 2.5 mg kg^{-1} i.v. of theophylline 48 h after the administration of corn oil or 3MC. To assess theophylline plasma concentrations, blood samples were withdrawn prior to and at 60, 90, 120, 180, 240, 300, 360, 420 and 480 min after its administration. Urine was collected for 24 h to assay theophylline metabolites.

Assays

To assess whether the changes in P450 activity induced by hypoxia and inflammation were secondary to pre- or post-translational changes in P450, the amounts of CYP1A1, 1A2 and 3A6 proteins were quantified by Western blot analysis in microsomal proteins of rabbits exposed to a 10% FiO_2 for 24 h ($n=3$), rabbits with an inflammatory reaction ($n=3$), and rabbits exposed to the combination of hypoxia and inflammation ($n=3$), rabbits which received 3MC ($n=3$) and rabbits which have been exposed to 3MC and hypoxia ($n=3$). Proteins were separated by polyacrylamide gel electrophoresis (7.5% polyacrylamide) under non-reducing conditions (Smith, 1994). Separated proteins were electrophoretically transferred to a nitro-cellulose membrane using a semidry 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.), and visualized with an alkaline phosphatase conjugated secondary antibody using nitro blue tetrazolium as the substrate (Kruger, 1994). CYP3A6 was detected with a monoclonal anti-rat CYP3A1 (Oxford Biochemical Research, Oxford, MI, U.S.A.) using a chemiluminescence reagent (horseradish peroxidase enzyme) conjugated secondary antibody and visualized by autoradiography (Thorpe *et al*., 1985). The intensities of the bands were measured with a software Alphaimager version 3.24.

Theophylline and metabolites in plasma and urine were assayed by HPLC as described elsewhere (du Souich *et al*., 1989). The precision of the assay for theophylline concentrations of 3 and 16 $\mu g\ ml^{-1}$ in plasma and urine was 2.1 and 2.3%; the precision for 3MX, 1MU and 1,3DMU at the concentration 16 $\mu g\ ml^{-1}$ in urine was 4.8, 5.7 and 6.7%, respectively. Protein content in the microsomal preparation was determined according the method of Lowry *et al*. (1951). The precision of the method for an average concentration of 200 $\mu g\ ml^{-1}$ of albumin was 3.4%. Seromucoids were measured as described elsewhere (Parent *et al*., 1992), and the precision of the assay for an average concentration of 25 $mg\ dl^{-1}$ was 5.3%.

Drugs and chemicals

Theophylline and its metabolites, 3MX, 1MU and 1,3DMU, 3MC, nitro blue tetrazolium and other chemicals were purchased from Sigma Chemical Company (St. Louis, MS, U.S.A.). Polyacrilamide was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). The alkaline phosphatase secondary antibody was acquired from Oxford Biochemical Research (Oxford, MI, U.S.A.), and the horseradish peroxidase enzyme was purchased from Amersham Life Sciences (Oakville, Ontario, Canada).

Analysis of data

The area under theophylline plasma concentration-time curve ($AUC_{0\rightarrow t}$) from zero to the last concentration measured (C_t) was estimated by means of the trapezoidal method. The $AUC_{0\rightarrow\infty}$ was obtained by adding to the $AUC_{0\rightarrow t}$ the value of C_t/z, where z is theophylline rate constant of disposition estimated from the slope of the terminal phase of theophylline plasma concentrations. Predicted apparent volume of distribution of theophylline at steady state (Vd_{SS}) was estimated according to noncompartmental analysis based on statistical moment theory (Gibaldi & Perrier, 1982) with the computer program Pharmacokinetic Data Analysis Program included in Lotus 1,2,3, Version 2.2 (Lotus Development Corporation, Cambridge, MD, U.S.A.). The fraction of theophylline metabolic clearance generating each metabolite was calculated as follows:

$$Cl_S = D/AUC_{0-\infty} \qquad (ml\ min^{-1}\ kg^{-1})$$
$$Cl_R = X_U/AUC_{0-\infty} \qquad (ml\ min^{-1}\ kg^{-1})$$
$$Cl_M = Cl_S - Cl_R \qquad (ml\ min^{-1}\ kg^{-1})$$

where Cl_S, Cl_R and Cl_M are theophylline systemic, renal and metabolic clearances, respectively. D is theophylline dose, and X_U is the amount of theophylline recovered in urine. The fraction of theophylline undergoing metabolism (F_TM) is defined by:

$$F_TM = Cl_M/Cl_S$$

The fraction of theophylline being metabolised to 3MX ($F_{3MX}M$), assuming that the metabolite is solely eliminated through the kidney, is calculated as follows:

$$F_{3MX}M = X_{U3MX}/(F_TM \cdot D)$$

where X_{U3MX} is the amount of 3MX recovered in urine. The formation clearance of 3MX or the fraction of theophylline metabolic clearance yielding 3MX (Cl_{M3MX}) is calculated using the following equation:

$$Cl_{M3MX} = F_{3MX}M \cdot Cl_M \qquad (ml\ min^{-1}\ kg^{-1})$$

Similar equations were used to calculate the formation clearance of 1MU and 1,3DMU. For these calculations, the values of plasma concentrations and urinary amounts were expressed in molar concentrations.

Comparison of the results obtained under the various experimental conditions with those in the control group was carried out using a one way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test (Winer, 1971). The minimal level of significance was $P<0.05$. All results are presented as mean $\pm$ standard error (s.e.).

Results

Effect of hypoxia on P450 activity and apoproteins

Exposure of the rabbits to a 10% FiO_2 atmosphere established a very stable hypoxemia, with a PaO_2 ranging from 33.2 ± 1.2 (mean $\pm$ s.e.mean) to 34.5 ± 1.6 mmHg; $PaCO_2$ decreased from 20.4 ± 0.9 to 12.9 ± 0.5 mmHg, with no repercussions on arterial pH, i.e. before hypoxia it was 7.526 ± 0.105 and remained fairly constant throughout the study at 7.566 ± 0.009. Compared with animals breathing air, hypoxia increased theophylline plasma concentrations in the terminal phase and as a consequence, theophylline $AUC_{0-\infty}$ increased by almost 20% (Table 1). The $AUC_{0-\infty}$ was enhanced because hypoxia decreased theophylline Cl_M, as reflected by the decrease in the formation clearance of the three metabolites (Figure 1). Compared with rabbits breathing air, hypoxia reduced the amount of CYP1A1 and 1A2 proteins, but increased that of 3A6 (Figure 2).

Effect of turpentine-induced inflammatory reaction on P450 activity and apoproteins

Forty eight hours after turpentine administration, a marked inflammatory reaction with a granuloma was apparent at the site of the injection. Rectal temperature increased from 39.1 ± 0.2 to $40.2\pm0.2^{\circ}C$ ($P<0.05$), as well as the seromucoids from 35.9 ± 5.7 to 82.5 ± 12.1 $mg\ dl^{-1}$ ($P<0.05$). The inflam-

Table 1 Influence of hypoxia ($PaO_2 = 33.3 \pm 1.2$ mmHg) on the kinetic parameters of theophylline injected intravenously (2.5 mm kg^{-1}) to conscious rabbits ($n = 8$/group)

	Control rabbits	*Hypoxic rabbits*
$AUC_{0\rightarrow\infty}$ (μg min ml^{-1})	1333 ± 321*	1592 ± 480**
Vd_{ss} (ml kg^{-1})	654 ± 64	693 ± 114
Cl_S (ml min^{-1} kg^{-1})	1.96 ± 0.43	1.69 ± 0.45**
Cl_R (ml min^{-1} kg^{-1})	0.23 ± 0.02	0.20 ± 0.03
Cl_M (ml min^{-1} kg^{-1})	1.73 ± 0.16	1.48 ± 0.13**

*mean ± s.e.mean. **$P < 0.05$ compared with the control group. $AUC_{0\rightarrow\infty}$ is the area under theophylline plasma concentrations as a function of time, from 0 to infinity; Vd_{ss} is predicted theophylline volume of distribution at steady state; Cl_S, Cl_R and Cl_M are theophylline systemic, renal and metabolic clearances.

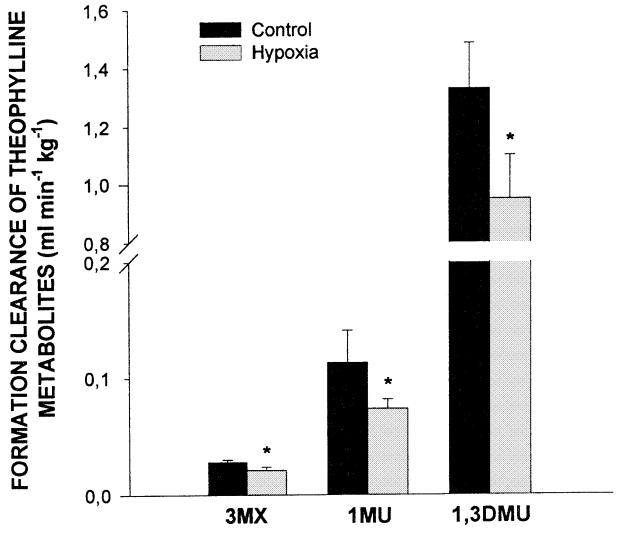

Figure 1 Fraction of theophylline metabolic clearance generating 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU) following the intravenous administration of 2.5 mg kg^{-1} of theophylline to control conscious rabbits, or rabbits with acute moderate hypoxia. Vertical bars are s.e.mean. *$P < 0.05$ compared with the control.

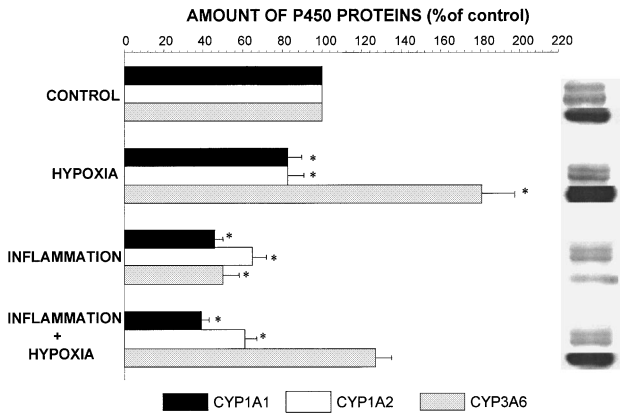

Figure 2 Relative amounts and bands of hepatic CYP1A1, 1A2 and 3A6 immunoreactive proteins assessed by Western immunoblot analysis in control rabbits, rabbits subjected to hypoxia ($PaO_2 \approx 35$ mmHg) for 24 h, rabbits with a turpentine-induced inflammatory reaction, and rabbits with hypoxia and a turpentine-induced inflammatory reaction. Vertical bars are s.e.mean. *$P < 0.05$ compared with control.

matory reaction increased theophylline terminal phase plasma concentrations (Figure 3), and consequently theophylline $AUC_{0\text{-}\infty}$ ($P < 0.05$) (Table 2). The increase in $AUC_{0\text{-}\infty}$ was secondary to a reduction in theophylline Cl_M. The inflammatory reaction decreased ($P < 0.05$) the formation clearance of 3MX and 1MU by 95 and 63%, respectively. Even if the amount of 1,3DMU collected in urine diminished by 36%, this difference did not reach statistical significance (Figure 4). Compared with control rabbits, the turpentine-induced inflammatory reaction reduced the amount of CYP1A1, 1A2 and 3A6 proteins by almost 50% (Figure 2). Renal clearance of theophylline was not altered by the inflammatory reaction (Table 2).

Combined effect of hypoxia and an inflammatory reaction on P450 activity and apoproteins

Rectal temperature as well as seromucoids were increased in rabbits with the inflammatory reaction, i.e. from 38.8 ± 0.3 to 40.1 ± 0.1°C ($P < 0.05$), and from 23.6 ± 8.4 to 51.6 ± 5.9 mg dl^{-1} ($P < 0.05$), respectively. Before the induction of hypoxia, PaO_2 was 89 ± 5 mmHg, and when theophylline was injected, the PaO_2 was 54 ± 1 mmHg; arterial pH remained rather stable all along the experiment (7.488 ± 0.011) as did the $PaCO_2$ (17.0 ± 0.6 mmHg). In hypoxic rabbits with an inflammatory reaction (Figure 3), theophylline plasma concentrations and $AUC_{0\text{-}\infty}$ were only slighly increased ($P > 0.05$) (Table 2). Compared with rabbits with a turpentine-induced inflammatory reaction, theophylline metabolic clearance was increased in rabbits with combined hypoxia and inflammation. Compared with control rabbits, in hypoxic rabbits with an inflammatory reaction, the formation clearance of 3MX and of 1MU decreased by 83 and 88%, respectively ($P < 0.05$), and that of 1,3DMU was not modified (≈5%) (Figure 4). In rabbits with combined hypoxia and inflammation, CYP1A1 and 1A2 proteins were about 50% the values observed in control rabbits, although the amount of CYP3A6

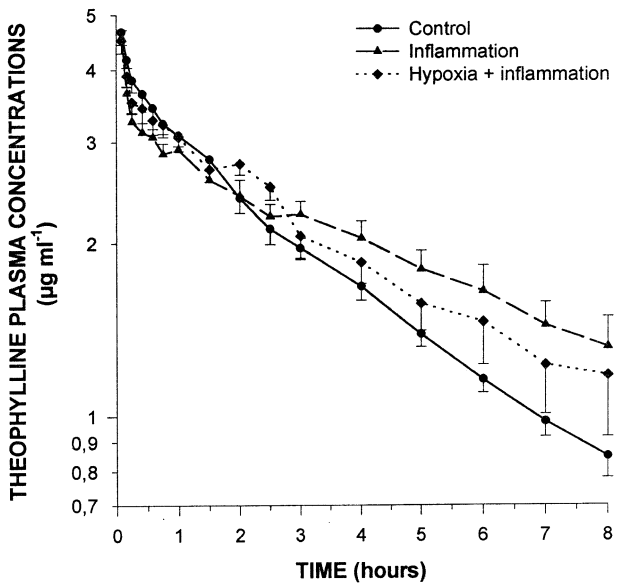

Figure 3 Plasma theophylline concentrations as a function of time following the intravenous administration of 2.5 mg kg^{-1} to control conscious rabbits, to rabbits with a turpentine-induced inflammatory reaction, and rabbits with an inflammatory reaction and acute moderate hypoxia ($PaO_2 \approx 55$ mmHg) for 24 h. Vertical bars are s.e.mean.

protein remained slightly greater than that observed in controls (Figure 2).

Combined effect of 3MC and hypoxia on P450 activity and apoproteins

Compared with the results obtained in rabbits receiving saline s.c., corn oil did not modify the kinetics of theophylline. In rabbits receiving 3MC, theophylline plasma concentrations and theophylline $AUC_{0-\infty}$ were lower than in controls (Table 3) due to an increase in theophylline Cl_M. The administration of 3MC enhanced the rate of production of all three metabolites (Figure 5). Hypoxia did not reverse the enzyme-induction produced by 3MC, i.e. theophylline $AUC_{0-\infty}$ was similar to that observed when the rabbits were exposed only to 3MC (Table 3). In rabbits receiving 3MC and subjected to hypoxia,

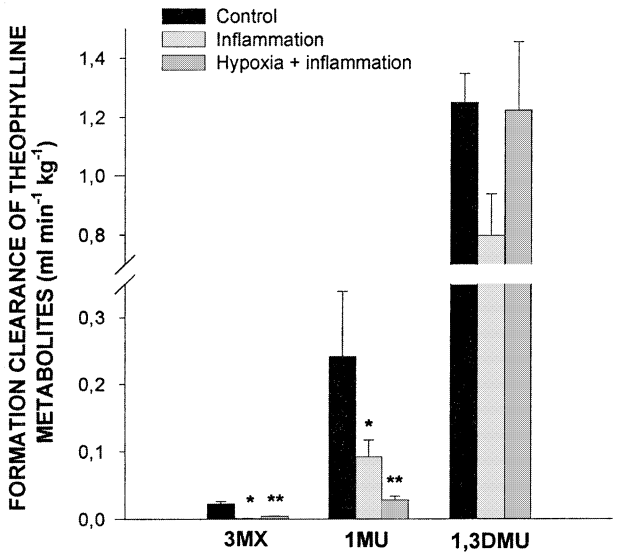

Figure 4 Fraction of theophylline metabolic clearance generating 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU) following the intravenous administration of 2.5 mg kg^{-1} of theophylline to controls conscious rabbits, rabbits with a turpentine-induced inflammatory reaction, and rabbits with an inflammatory reaction and acute moderate hypoxia ($PaO_2 \approx 55$ mmHg) for 24 h. Vertical bars are s.e.mean. *, **$P<0.05$ compared with the control or with inflammation alone, respectively.

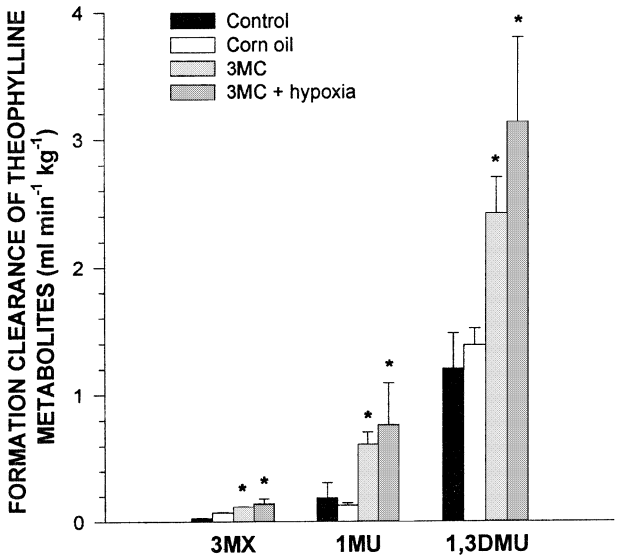

Figure 5 Fraction of theophylline metabolic clearance generating 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU) following the intravenous administration of 2.5 mg kg^{-1} of theophylline to control conscious rabbits, to control rabbits receiving corn oil s.c., to rabbits receiving 3-methylcholanthrene (3MC) (80 mg kg^{-1} s.c.) or to rabbits receiving 3MC and subjected to acute moderate hypoxia ($PaO_2 \approx 35$ mmHg) for 24 h. Vertical bars are s.e.mean. *$P<0.05$ compared with the control or with 3MC alone, respectively.

Table 2 Effect of a turpentine-induced inflammatory reaction alone or associated with hypoxia on the kinetics of intravenous theophylline (2.5 mg kg^{-1}) in conscious rabbits

	Control ($n=8$)	*Turpentine* ($n=8$)	*Hypoxia + turpentine* ($n=6$)
$AUC_{0\rightarrow\infty}$ (μg min ml^{-1})	1182±68*	1930±319**	1595±307
Vd_{ss} (ml kg^{-1})	687±38	805±37	713±24
Cl_S (ml min^{-1} kg^{-1})	2.16±0.12	1.55±0.25**	1.85±0.30
Cl_R (ml min^{-1} kg^{-1})	0.24±0.08	0.17±0.03	0.16±0.02
Cl_M (ml min^{-1} kg^{-1})	1.92±0.12	1.38±0.24**	1.69±0.30***

*mean±s.e.mean. **$P<0.05$ compared to control values. ***$P<0.05$ compared to turpentine values. $AUC_{0\rightarrow\infty}$ is the area under theophylline plasma concentration as a function of time; Vd_{ss} is theophylline predicted volume of distribution at steady state; Cl_S, Cl_R and Cl_M are theophylline total body, renal and metabolic clearances, respectively.

Table 3 Influence of 3-methylcholanthrene (3MC) (80 mg kg^{-1}) alone or combined to hypoxia on the kinetic parameters of intravenous theophylline (2.5 mg kg^{-1}) in conscious rabbits ($n=7$/group)

	Control	*Corn oil*	*3MC*	*3-MC hypoxia*
$AUC_{0\rightarrow\infty}$ (μg min ml^{-1})	1579±216*	1157±77	672±36**	576±46**
Vd_{ss} (ml kg^{-1})	526±37	626±24	591±14	696±28
Cl_S (ml min^{-1} kg^{-1})	1.84±0.35	2.22±0.15	3.76±0.25**	4.95±0.43**
Cl_R (ml min^{-1} kg^{-1})	0.21±0.05	0.21±0.03	0.25±0.03	0.23±0.04
Cl_M (ml min^{-1} kg^{-1})	1.64±0.30	2.02±0.13	3.51±0.23**	4.29±0.35**

*mean±s.e.mean. **$P<0.05$ compared with values estimated in the control-corn oil group. $AUC_{0\rightarrow\infty}$ is the area under theophylline plasma concentration as a function of time, from 0 to infinity; Vd_{ss} is theophylline predicted volume of distribution at steady state; Cl_S, Cl_R and Cl_M are theophylline total body, renal and metabolic clearances.

theophylline Cl_M was increased by 112%, and as a consequence, the formation clearance of all three metabolites was enhanced (Figure 5). Relative to the amount of CYP1A1 and 1A2 proteins in control rabbits, 3MC increased the amount of these proteins by around 23 and 20% ($P<0.05$), respectively, and the combination of 3MC and hypoxia enhanced the amount of these apoproteins by 47 and 25%, respectively. On the other hand, 3MC and the combination of 3MC and hypoxia tended ($P>0.05$) to reduce the amount of CYP3A6 protein to 88 and 74% the values observed in controls, respectively (Figure 6).

Discussion

Moderate hypoxia reduced the formation clearance of 3MX, 1MU and 1,3DMU, and decreased the amount of CYP1A1 and 1A2 proteins, but increased CYP3A6 protein, implying that in the rabbit, CYP3A6 has a minor role in the formation of 1,3DMU, and that CYP1A1 and 1A2, as well as other P450 apoproteins, are the main source of 1,3DMU. Similar conclusion has been reached concerning the relative importance of CYP1A1, 1A2 and 3A4 in the metabolism of theophylline in humans (Tija *et al*., 1996). In addition, these results show that hypoxia affects selectively some P450 apoproteins. Several explanations may be invoked to understand the hypoxia-induced down-regulation of CYP1A1 and 1A2. Under hypoxic conditions, the organism reduces energy turnover and improves energetic efficiency, and to this purpose, ATP turnover is depressed (Hayashi *et al*., 1997), and ATP-demanding processes, such as protein synthesis and degradation are diminished (Hochachka *et al*., 1997). Taking into account the turnover of CYP1A1 and 1A2 proteins, with half-lives of 16 and 10 h (Shiraki & Guengerich, 1984), a decrease in the expression of these proteins is compatible with the results reported in the present study.

In vivo, hypoxia induces a time-dependent decrease in reduced glutathione (GSH) and other cellular antioxidants, and an increase in lipid peroxidation end-products in the liver (El-Bassiouni *et al*., 1998) as a result of the formation of reactive oxygen intermediates (ROI), such as superoxide anion ($O_2^{\bullet -}$) (Minor *et al*., 1993; Caraceni *et al*., 1995), hydrogen peroxide (H_2O_2) (Matuschak *et al*., 1996), and nitric oxide ($NO^{\bullet}$) (Gess *et al*., 1997). Theoretically, ROI could degrade P450 apoproteins directly as suggested by the fact that H_2O_2 formed in the hemoprotein active centre may interact with the enzyme associated Fe^{2+} leading to heme destruction and enzyme inactivation (Karuzina & Archakoz, 1994). Supporting a direct role of ROI in the P450 depression, is the fact that ROI generator systems are able to reduce the activity of the P450 (Khatsenko *et al*., 1993; Proulx & du Souich, 1995a). On the other hand, $O_2^{\bullet -}$ and H_2O_2 can depress the expression of selected apoproteins of hepatic P450 (Flowers & Miles, 1991; Barker *et al*., 1994). $NO^{\bullet}$ can inactivate reversibly (Kim *et al*., 1995) and irreversibly P450 apoproteins (Minamiyama *et al*., 1997), and can depress the mRNA encoding for p450 apoproteins (Khatsenko & Kikkawa, 1997). The fact that a single dose of 10000 IU of vitamin A prevents the effect of hypoxia on the P450 further supports the hypothesis that ROI are involved in the hypoxia-induced P450 down-regulation (Grover *et al*., 1985).

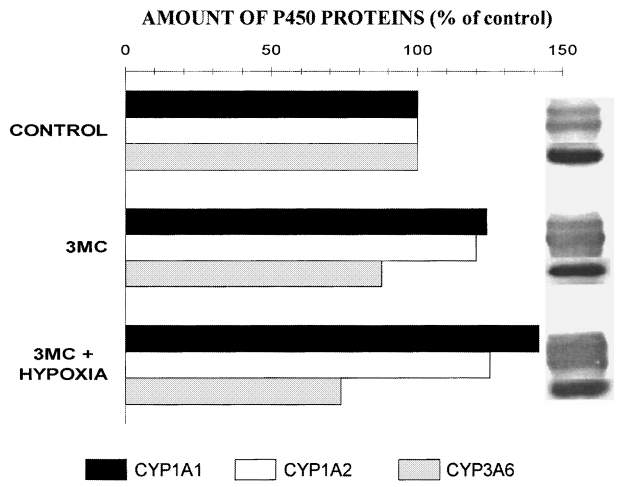

Figure 6 Relative amounts and bands of hepatic CYP1A1, 1A2 and 3A6 immunoreactive proteins assessed by Western immunoblot analysis in control rabbits, and rabbits that received 3-methylcholanthrene (3MC) alone (80 mg kg^{-1}) or combined with hypoxia ($PaO_2 \approx 35$ mmHg) for 24 h.

Moderate hypoxia for 24 h increases hepatic CYP3A6, a result that is in agreement with the report of Matsubayashi *et al*. (1997) showing that hypoxia enhances the rate of metabolism of bromocriptine, a substrate of CYP3A subfamily. Taking into account that CYP3A subfamily catalyses the formation of $NO^{\bullet}$ (Kuo *et al*., 1995), and that hypoxia stimulates the formation of $NO^{\bullet}$ (Gess *et al*., 1997) in the liver and in peripheral blood vessels (Fujimoto & Itoh, 1997), we might speculate that in response to hypoxia, the expression of CYP3A subfamily is enhanced to increase the blood flow to selected territories. Further studies must be conducted to confirm such hypothesis.

It has been shown that hypoxia enhances the concentration of circulating cytokines by increasing the outflow of TNF-α, IL-1α, and IL-1β from the liver (Wibbenmeyer *et al*., 1995; Matuschak *et al*., 1996), and TNF-α and IL-1α from the lung (Matuschak *et al*., 1998). *In vivo*, despite the fact that IL-1β, TNF-α, IL-6 and IFN-γ elicit a differential effect on P450 apoproteins, they depress CYP1A1 and 1A2 (Morgan, 1997). On the other hand, in rabbit's cultured hepatocytes, IL-1β and IL-2 reduce induced CYP3A6, and INF-γ is able to decrease the amounts of constitutive and induced CYP3A6 protein (Calleja *et al*., 1998), suggesting that these cytokines may not intervene in the hypoxia-induced increase in CYP3A6 protein. Since cytokines induce the production of ROI (Ghezzi *et al*., 1985; Feng *et al*., 1995) and the formation of $NO^{\bullet}$ in hepatocytes (Spitzer, 1994), it is tempting to postulate that hypoxia-induced cytokines are the mediators leading to the down-regulation of CYP1A1 and 1A2.

Turpentine-induced inflammatory reaction reduced theophylline Cl_M secondary to the down-regulation of CYP1A1, 1A2 and 3A6. The exact mechanism underlying the turpentine-induced P450 down-regulation is unknown but it is probably associated to a pre-translational mechanism (Morgan, 1989). The P450 depression induced by the inflammatory reaction is mediated by serum factors (El-Kadi *et al*., 1997; El-Kadi & du Souich, 1998) assumed to be cytokines (Morgan, 1997). As discussed above, cytokines can trigger the production of ROI which may down-regulate P450 apoproteins. Supporting such hypothesis, the turpentine-induced inflammatory reaction is associated with an intrahepatic increase in lipid peroxidation, decrease in catalase, superoxide dismutase and glutathione peroxidase activities, and a reduction in the levels of GSH (Proulx & du Souich, 1995b).

The combination of hypoxia and the inflammatory reaction does not elicit an additive effect on the P450, but rather the

effect of each experimental condition is reduced. Since the formation of 1,3DMU is not affected and CYP1A1 and 1A2 proteins are depressed, we may assume that the combination of hypoxia and inflammation protects or even increases other P450 apoproteins implicated in the formation of 1,3DMU. Several mechanisms may be invoked to explain the decrease in effect of each experimental condition. On the one hand, T-cells are necessary for the P450 down-regulation induced by an inflammatory reaction (Topfer *et al*., 1995), and hypoxia inhibits T-cell function (Meehan, 1987). In addition, hypoxia down-regulates *E. coli*-induced TNF-α, IL-1α and IL-1β hepatic genes, and so decreases the expression of TNF-α, IL-1α and IL-1β proteins (Wibbenmeyer *et al*., 1995; Matuschak *et al*., 1996). On the other hand, hypoxia prevents the pneumonia-induced activation of lipid peroxidation in liver mitochondria (Semenov & Iarosh, 1991), and attenuates the H_2O_2-induced mechanical and metabolic tissue damage (Hara & Abiko, 1995). Therefore, hypoxia may not only reduce the plasma mediators required to down-regulate the P450, but may also diminish intracellular events leading to the P450 down-regulation triggered by the inflammatory reaction. On the other hand, whenever hypoxia-induced CYP3A6 is involved in the formation of $NO^{\bullet}$ (Kuo *et al*., 1995) and $NO^{\bullet}$ is involved in the hypoxia-induced P450 depression (Minamiyama *et al*., 1997; Khatsenko & Kikkawa, 1997), the inflammatory reaction, by decreasing CYP3A6, should limit the effect of hypoxia. Further studies are required to understand the exact mechanism involved in the hypoxia-inflammation interaction.

3MC increases the amount of CYP1A1 and 1A2 proteins and prevents hypoxia-induced down-regulation of the P450. These results agree with reports showing that the P450 down-regulation induced by endotoxins, carrageenan and INF-γ, IL-1α, IL-1β, and IL-6 is prevented by the administration of 3MC (Ishikawa *et al*., 1992; Clark *et al*., 1995). Several explanations, not mutually exclusive, could be put forward to understand the present results. In the liver acinus, constitutive P450 apoproteins are essentially concentrated in the perivenous zone, where tissue oxygen tension is around 4%, with decreasing concentrations of P450 in midzonal and periportal zones, where tissue oxygen reaches tensions of 13% (Oinonen & Lindros, 1998). Endotoxins and cytokines produce a preferential perivenous cell injury (Ohno & Maier, 1995), where the production of ROI is greater than in periportal zones (Kukielka & Cederbaum, 1995). We might postulate that hypoxia affects more importantly P450 apoproteins in the perivenous zone that in the periportal zone. On the other hand, 3MC produces a marked increase of CYP1A1 and 1A2, and a 10% decrease of other P450 apoproteins in perivenous hepatocytes (Tanaka *et al*., 1997). Since in the present study, 3MC was injected 24 h before the rabbits were exposed to hypoxia, we may speculate that the induction produced in the perivenous zone masks any deleterious effect of hypoxia. Alternatively, 3MC depresses CYP2B1, 2C11 and 2E1 and oxidase activity of hepatic P450 (Sakai *et al*., 1992), apoproteins known to catalyse H_2O_2 and other ROI and to induce lipid peroxidation (Sakai *et al*., 1992; Ohmori *et al*., 1993); as a consequence, the ability of hypoxia to generate ROI and to depress CYP1A1 and 1A2 would be attenuated. Finally, 3MC may stabilize selected apoproteins because of its tight binding to them (Huang *et al*., 1986).

In conclusion, *in vivo* acute moderate hypoxia reduces the amount of CYP1A1 and 1A2 proteins, although it increases that of CYP3A6. On the other hand, a turpentine-induced inflammatory reaction decreases the amount of CYP1A1, 1A2 and 3A6 proteins. The combination of hypoxia and an inflammatory reaction elicits a very limited effect on the *in vivo* metabolism of theophylline, despite the fact that the amounts of CYP1A1 and 1A2 proteins remain comparable to that measured during the inflammatory reaction alone. From a practical point of view, the present results may explain why in patients with advanced complicated COLD, i.e. with hypoxia and chronic bronchial inflammatory reactions, the metabolism of theophylline is scarcely affected by the disease, and therapy with oxygen does not enhance theophylline metabolism (Cusack *et al*., 1986; du Souich *et al*., 1989). Indeed such extrapolation to human pathology must be done with caution because of species, pathology, and P450 apoprotein specificity of the cytokines. The pre-treatment of the rabbits with 3MC increased CYP1A1 and 1A2 preventing the down-regulation of these apoproteins induced by hypoxia.

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