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Acute Moderate Hypoxia in Conscious Rabbits: Effect on Hepatic Cytochrome P450 and on Reactive Oxygen Species

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Abstract

This study aimed to document whether acute moderate hypoxia modifies the amount and activity of hepatic cytochrome P450 and in addition, induces changes in the production or the ability to neutralize oxygen reactive species (ORS).

Rabbits were exposed to a low partial pressure of oxygen (12%) for 8 or 24 h, killed, and the amount and activity of cytochrome P450, lipid peroxidation, microsomal chemiluminescence and enzymatic scavenger activity were assessed in the liver. After 8 h of hypoxia, total amount but not the activity of cytochrome P450 was decreased, although after 24 h of hypoxia, both the amount and the activity of cytochrome P450 were decreased. Hypoxia for 8 h increased the activity of glutathione peroxidase. However, after 24 h of hypoxia, lipid peroxidation, microsomal chemiluminescence and superoxide dismutase activity were increased, while hepatic glutathione and glutathione peroxidase activity were reduced, modifications that suggest an enhanced presence of ORS. In in-vitro studies, an ORS generating system reduced the activity of cytochrome P450 and enhanced lipid peroxidation of hepatic microsomal membranes, supporting the view that ORS can impair cytochrome P450.

The results of the present study show that hypoxia induces changes in the amount and activity of cytochrome P450, as well as in the production or the ability to neutralize ORS, and that these changes are time-dependent.

The metabolic clearance of selected drugs, such as theophylline, is decreased in patients with chronic obstructive lung disease (Hendeles et al 1977), cor pulmonale (Vicuna et al 1979), acute pulmonary oedema (Piafsky et al 1977) and congestive heart failure (Powell et al 1978; Kuntz et al 1983) when complicated by an episode of acute hypoxia, a common event with these patients. Supporting the hypothesis that hypoxia modulates the rate of drug biotransformation, is the fact that in conscious healthy rabbits, acute moderate hypoxia reduces the clearance of theophylline (Letarte & du Souich 1984), phenytoin (du Souich et al 1986), and lignocaine (Marleau et al 1987).

It has been reported that hypoxia decreases the activity and amount of cytochrome P450 (Srivastava et al 1980; Jordi-Racine et al 1988). However, the data published in the literature is contradictory. For instance, in rats exposed to an atmosphere with a relative amount of oxygen (FiO_2) of 8–10% for 1 h, the amount and activity of cytochrome P450 was increased (Shugalei et al 1991, 1992). In other studies, where rats were exposed to an FiO_2 of 12% for 2 h, the amount of cytochrome P450 was not modified (Shen et al 1982; Grek et al 1984). On the other hand, in mice and rats exposed to an FiO_2 of 8 or 10·5% for 10 days, hepatic cytochrome P450 was decreased or unchanged, respectively (Bechtel et al 1976; Shan et al 1992), and in rats exposed to hypobaric hypoxia for 4–5 or 5–12 weeks, the amount of cytochrome P450 was not affected or decreased, respectively (Bonkovsky et al 1986; Costa 1990). On the basis of these reports it is difficult to conclude that hypoxia reduces the amount of hepatic cytochrome P450.

In patients with an acute episode of asthma and hypoxia, the de-novo production of oxygen reactive species (ORS) by airspace cells is increased (Jarjour & Calhoun 1994). Using animal models, the effect of hypoxia on ORS production or neutralization remains unclear. For instance, in rats with acute hypoxia for 1 h, the amount of conjugated dienes increased, while the activity of superoxide dismutase (SOD) and that of catalase decreased (Shugalei et al 1992). On the other hand, in another study, after 10 days of hypoxia, SOD, epoxide hydrolase and catalase activities remained unchanged (Shan et al 1992). However, when the rats were subjected to chronic hypobaric hypoxia, the activities of SOD, catalase and glutathione peroxidase were reduced (Costa 1990). These studies do not allow us to establish a time-dependant relationship between hypoxia and changes in activity of enzymatic scavengers, and, furthermore, cannot exclude the possibility that the results are influenced by hypobaria.

The aims of this study were to investigate the effect of acute normobaric moderate hypoxia on hepatic cytochrome P450, and its ability to modify the production or neutralization of ORS.

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Materials and Methods

In-vivo studies

Hypoxia model. Male New Zealand rabbits (Ferme Cunipur, St-Valérien, Québec), 2·0–2·2 kg, were used throughout. After

a week of acclimatization in ambient air, a first group of nine rabbits was exposed to an atmosphere with a relative amount of oxygen (FiO_2) of 12% for 8 h. A second group of seven animals was exposed to a 12% FiO_2 for 24 h. Two groups of ten rabbits breathing laboratory air were used as controls.

To induce hypoxia in rabbits, the animals were placed in a Plexiglas chamber (0·75 × 1·20 × 1·25 m), where the desired FiO_2 (12%) was regulated with an oxygen monitor (OM-15, Sensor Medics Corporation, CA) connected to an electrovalve (Asco Valves, Brantford, Ontario) that allowed the access of nitrogen into the chamber displacing the air from the chamber. The value of 12% FiO_2 was selected to obtain an arterial partial pressure of O_2 (PaO_2) of approximately 50 mmHg. Humidity in the chamber was maintained at 50% by the recirculation of the air through a refrigerating system. The temperature was kept at 22–24°C. All animals had free access to Purina Laboratory Chow and water during the acclimatization period, and during the 8 or 24 h of the experiment. Control rabbits were also placed into the chamber for the experiments, but were allowed laboratory air at an FiO_2 of 21%. Arterial blood samples were withdrawn at various intervals of time during the experiments to control blood gases and pH with a gas analyser (Model IL Micro 13-03/213-05, Instrumentation Laboratory, Lexington, MA). Eight or 24 h after initiating the hypoxia, the rabbits were killed and the liver was removed to isolate the microsomal and supernatant fractions.

Processing of hepatic microsomal and supernatant fractions. A portion of the liver was washed with saline at 4°C and excised. After homogenization and centrifugation at 105 000 *g*, the resulting supernatant was chromatographed on a Sephadex G-25 column to remove endogenous substrates able to decrease the activity of the xanthine oxidase system (EC. 1.1.3.22) (Engerson et al 1987). The eluate was used to assay the activity of xanthine oxidase + dehydrogenase. Another aliquot of the liver was processed as described by Sinclair et al (1990) to measure by chemiluminescence the de-novo formation of ORS in the microsomal fraction. The supernatant and microsomal fractions of the remaining part of the liver were obtained according to the method of Cinti et al (1972) and used to assess glutathione peroxidase (EC. 1.11.1.9), catalase (EC. 1.11.1.6), CuZn superoxide dismutase (EC. 1.15.1.1), and cytochrome P450. The supernatant and microsomal fractions were stored at −80°C until assayed.

Assays. Protein content in the hepatic supernatant and microsomal fractions was determined by the method of Lowry et al (1951). Total microsomal cytochrome P450 was assessed by the method of Omura & Sato (1964). Hepatic cytochrome P450 activity was documented by measuring the rate of hydroxylation of aniline by estimating the formation of *p*-aminophenol (Brodie & Axelrod 1948), the rate of *N*-demethylation of aminopyrine by measuring the formation of formaldehyde (Pederson & Aust 1970), and the rate of *O*-de-ethylation of 7-ethoxycoumarin, measuring the formation of hydroxycoumarin (Jacobson et al 1974).

To demonstrate whether hypoxia increases the concentration of ORS in hepatic tissues, several indirect markers were measured. Being malondialdehyde hydrosoluble, lipid peroxidation was assessed in the supernatant by measuring the amount of malondialdehyde by means of the thiobarbituric acid reaction (Ohkawa et al 1979; Valenzuela 1991). Since hepatic cytochrome P450 is a potential source of ORS, the possibility of de-novo production of ORS was assessed in the microsomal fraction measuring the formation of ORS induced by the oxidation of NADPH (Weimann et al 1984; Archakov & Bachmanova 1990), by chemiluminescence (Sinclair et al 1990).

An increase in ORS could be also secondary to a decrease in the activity of scavenger systems. Therefore, glutathione peroxidase activity was evaluated measuring the rate of disappearance of NADPH (Schisler & Singh 1988). The activity of catalase was assayed using the method of Clairborne (1985). The activity of cytosolic CuZn superoxide dismutase was evaluated using the procedure described by Flohé & Otting (1984). Since mitochondrial fraction was removed by ultracentrifugation, the manganese form of superoxide dismutase was not evaluated. Reduced glutathione was measured in the supernatant using the dithionitrobenzoic acid reaction (Benke et al 1974). This method measures thiol groups from non-protein and from protein sulphydryl groups, but since proteins were precipitated and discarded, and glutathione constitutes as much as 95% of the non-protein thiol pool in hepatic cells (Russo & Bump 1988), the method used measures essentially glutathione only. Xanthine oxidase is another source of ORS; therefore its activity was assayed in the supernatant, using the procedure described by McKelvey et al (1988).

In-vitro studies

The ability of ORS to decrease cytochrome P450 activity was assessed in-vitro by incubating microsomal membranes with an ORS-generating system incorporating xanthine-xanthine oxidase, EDTA iron(III) sodium salt and ferric sulphate. Briefly, the microsomal fraction of the liver from control rabbits (n = 6) was suspended in 10 mM phosphate buffer pH 7·4, and incubated with 0·33 M xanthine, 0·52 units mL^{-1} xanthine oxidase, 0·1 mM EDTA iron(III) sodium salt, 0·1 mM ferric sulphate and 0·043 M NADPH, at 37°C for 45 min (Byczkowski & Gessner 1987). Lipid peroxidation and cytochrome P450-dependent monooxygenase activity were measured.

Statistical analysis

Comparison of the results from the various experimental groups and their controls was carried out using Student's *t*-test for unpaired data, with a significance criteria of $P < 0·05$ (Winer 1971).

Results

In-vivo studies

The experimental conditions generated a stable hypoxia, with a mean arterial PaO_2 of 48 ± 1 mmHg (± s.e.), compared with a mean PaO_2 of 86 ± 2 mmHg in control animals breathing laboratory air. Arterial $PaCO_2$ and pH remained constant with average values of 20 ± 2 mmHg and 7·57 ± 0·03, respectively, compared with 20 ± 1 mmHg and 7·53 ± 0·02 in control animals.

Table 1. Total amount and activity of hepatic microsomal cytochrome P450-dependent mono-oxygenase in rabbits exposed to acute moderate hypoxia for 24 h.

Parameters	Control (n = 10)	Hypoxia (n = 7)
Cytochrome P450 (nmol (mg protein)$^{-1}$)	0·85 ± 0·06	0·46 ± 0·05*
Hydroxylation of aniline (nmol p-aminophenol-HCl min^{-1} (mg protein)$^{-1}$)	0·50 ± 0·03	0·36 ± 0·04*
N-Demethylation of aminopyrine (nmol formaldehyde min^{-1} (mg protein)$^{-1}$)	6·19 ± 0·41	3·74 ± 0·43*
O-De-ethylation of 7-ethoxycoumarin (nmol 7-hydroxycoumarin min^{-1} (mg protein)$^{-1}$)	0·46 ± 0·04	0·32 ± 0·02*

Mean ± s.e. *$P < 0·05$ compared with corresponding control.

Eight hours of hypoxia was enough to increase lipid peroxidation by 14%, from 0·45 ± 0·01 to 0·52 ± 0·03 nmol malondialdehyde (mg protein)$^{-1}$ ($P < 0·05$), and decrease the total amount of cytochrome P450 by 18%, from 0·84 ± 0·08 to 0·68 ± 0·05 nmol (mg protein)$^{-1}$ ($P < 0·05$), without significantly affecting its activity. Compared with control animals, the activity of glutathione peroxidase was 13% higher in hypoxic rabbits, i.e. 406 ± 13 compared with 358 ± 14 nmol NADPH consumed min^{-1} (mg protein)$^{-1}$ ($P < 0·05$), whereas the activities of catalase and CuZn superoxide dismutase were not modified.

Twenty-four hours of hypoxia reduced the total amount of cytochrome P450 by 45% (Table 1). The hydroxylation of aniline, the *N*-demethylation of aminopyrine and the *O*-de-ethylation of 7-ethoxycoumarin were reduced by 27, 39, and 31%, respectively. Compared with controls, 24 h of hypoxia increased lipid peroxidation by 34% (Table 2). In this group of animals, hypoxia almost doubled the chemiluminescence in the microsomal fraction. Xanthine oxidase activity was not modified by hypoxia, giving values of 0·43±0·13 and 0·47±0·08 nmol uric acid min^{-1} (mg protein)$^{-1}$ respectively in control and hypoxic rabbits.

Table 2. Lipid peroxidation, chemiluminescence and amount or activity of scavengers in liver from rabbits exposed to acute moderate hypoxia for 24 h

Parameters	Control (n = 10)	Hypoxia (n = 7)
Lipid peroxidation (nmol malondialdehyde (mg protein)$^{-1}$)	0·57 ± 0·03	0·77 ± 0·03*
Microsomal chemiluminescence (mV (mg protein)$^{-1}$)	475 ± 58	923 ± 102*
Glutathione (reduced form) (μmol (g wet tissue)$^{-1}$)	10·4 ± 0·4	6·8 ± 0·9*
Glutathione peroxidase (nmol NADPH consumed min^{-1} (mg protein)$^{-1}$)	502 ± 23	278 ± 33*
Superoxide dismutase (Cu-Zn) (units min^{-1} (mg protein)$^{-1}$)	12·3 ± 0·4	16·1 ± 0·6*
Catalase (μmol H_2O_2 consumed min^{-1} (mg protein)$^{-1}$)	82 ± 4	98 ± 10

Mean ± s.e. *$P < 0·05$ compared with corresponding control.

Table 3. In-vitro effect of ORS (oxygen reactive species) on hepatic microsomes

Parameters	ORS-generating system	
	Without	With
Lipid peroxidation (nmol malondialdehyde (mg protein)$^{-1}$)	11·01 ± 0·61	23·97 ± 0·18*
Hydroxylation of aniline (nmol *p*-aminophenol-HCl min^{-1}(mg protein)$^{-1}$)	0·31 ± 0·01	0·16 ± 0·01*
N-demethylation of aminopyrine (nmol formaldehyde min^{-1} (mg protein)$^{-1}$)	4·48 ± 0·04	3·15 ± 0·05*
O-De-ethylation of 7-ethoxycoumarin (nmol 7-hydroxycoumarin min^{-1} (mg protein)$^{-1}$)	0·77 ± 0·03	0·40 ± 0·04*

Values are the mean ± s.e. for six microsomal hepatic fractions from different rabbits. *$P < 0·05$ compared with corresponding control.

Compared with animals breathing laboratory air, the activity of CuZn superoxide dismutase was increased by 31% in the group of animals exposed to a low FiO_2 (Table 2). On the other hand, the activity of glutathione peroxidase was decreased by 45%. Interestingly, relative to control animals, in the liver of hypoxic rabbits, the reduced form of glutathione was decreased by 35%. Catalase activity was not affected by 24 h of hypoxia.

In-vitro studies

After 45 min incubation of the hepatic microsomes with the ORS-generating system, microsomal lipid peroxidation was more than doubled (Table 3). The activity of cytochrome P450 appeared seriously affected, since the rates of aniline hydroxylation, aminopyrine *N*-demethylation, and 7-ethoxycoumarin *O*-de-ethylation were decreased by 47, 30 and 48%, respectively.

Discussion

The results of the present in-vivo studies show that in rabbits subjected to normobaric acute moderate hypoxia there are time-dependent changes in the amount of hepatic cytochrome P450 and its activity, as well as in several markers denoting the presence of ORS. Eight hours of acute moderate hypoxia reduced slightly the amount of cytochrome P450 without affecting its activity, and on the other hand, increased lipid peroxidation as well as the activity of glutathione peroxidase. Twenty-four hours of hypoxia produced a substantial reduction in total amount and

activity of cytochrome P450, decreased reduced glutathione and the activity of glutathione peroxidase, and increased that of superoxide dismutase, changes that reflect higher concentrations of ORS.

After 24 h of hypoxia, the reduction in total amount of cytochrome P450 may explain the decrease in the activities of aniline hydroxylase, aminopyrine demethylase and 7-ethoxycoumarin dealkylase. It should be noted that the decrease in total amount of cytochrome P450 is essentially due to a reduction in several isoenzymes, among them those with molecular weights of 50, 52 and 55 kDa (du Souich et al 1990), which have been associated with CYP 2B1, CYP 2E1 and CYP 1A1 (Gonzalez 1992; Guengerich 1992). Confirming that only selected isozymes are affected by hypoxia, Lindstrom et al (1990) using the hepatic ischaemia-reperfusion model, reported time-dependent impairment of cytochrome P450, accompanied by a reduction in the rates of ethylmorphine demethylation and ethoxyresorufin dealkylation, while the rate of pentoxyresorufin dealkylation was not affected, and concluded that certain isoenzymes are more susceptible to ischaemia-reperfusion injury.

The increase in hepatic lipid peroxidation and in the de-novo formation of ORS, as well as the changes in activity of hepatic scavenger enzymes during acute hypoxia, allow us to confirm that hypoxia increased hepatic concentration of ORS, and to speculate on the nature and the source of the ORS. After 8 h of hypoxia, glutathione peroxidase activity increased in hepatic tissues, possibly in response to greater amounts of ORS, conceivably organic hydroperoxides. The activities of superoxide dismutase and catalase were not modified at this stage. After 24 h of hypoxia, the activity of glutathione peroxidase, as well as the concentration of reduced glutathione decreased. Longer periods of hypoxia, i.e. 10 or more days, are also accompanied by a reduction in the activity of glutathione peroxidase (Costa 1990; Shan et al 1992). On the other hand, after 24 h of moderate hypoxia the activity of superoxide dismutase increased, possibly reflecting enhanced formation of superoxide. Catalase, usually operative in the presence of high concentrations of hydrogen peroxide, and not organic hydroperoxides, remained unchanged. Therefore, according to the changes in activity of scavenger enzymes, we may postulate that the increase in ORS is secondary, firstly, to an overproduction of these reactive compounds, and thereafter, to a decrease in the protective effect of glutathione peroxidase, together with an increase in superoxide dismutase activity.

The decline in glutathione peroxidase activity observed in-vivo after 24 h of hypoxia could be associated with the decreased availability of reduced glutathione. The depletion in glutathione could be caused by hypoxia, since it has been demonstrated that in rat isolated hepatocytes, a low FiO_2 decreases the synthesis rate and the cellular content of glutathione (Jones 1985; Shan et al 1992). The depletion in glutathione could also be secondary to a drop in NADPH, the substrate required to reduce oxidized glutathione to the reduced form (Schisler & Singh 1988), since it has been demonstrated that low cellular oxygen levels (≈ 15 μM) for short periods of time (30 min) are sufficient to decrease the synthesis of NADPH (Tribble & Jones 1990). In the present study, the rabbits had an average PaO_2 of 48 mmHg for 24 h; therefore, it is possible to predict that oxygen concentration in hepatic cells, particularly centrilobular, should be lower than 15 μM (de Groot et al 1988). Under hypoxic conditions, the availability of ATP is reduced because of a decrease in glucose-6-phosphate dehydrogenase activity (Shan et al 1992), or of an exhaustion of cytochrome oxidase activity and glycolysis (Trump et al 1974; Aw & Jones 1982), and as a consequence, NADPH synthesis will be reduced. On the other hand, it has been reported that a decreased availability of ATP reduces the synthesis of glutathione from methionine, by limiting *S*-adenosylmethionine synthetase activity, a mechanism that may also contribute to the depletion of glutathione (Shan et al 1989).

The source of ORS in the liver during hypoxia remains unknown. Using ischaemia-reperfusion or perfused-organ models, it has been shown that anoxia enhances the activity of the xanthine oxidase system (Engerson et al 1987). Under the present experimental conditions, conscious rabbits with normobaric acute moderate hypoxia compatible with life, the activity of the xanthine oxidase system was not modified; as a consequence, the xanthine oxidase system is an improbable source of overproduction of ORS. Alternatively, cytochrome P450 can generate singlet oxygen (Cadenas et al 1983). Supporting the view that during hypoxia cytochrome P450 could be a source of ORS is the fact that chemiluminescence in microsomal membranes was increased after 24 h of hypoxia. Further studies are required to determine the exact source of the reactive oxygen species.

The decrease of selected isoenzymes of the cytochrome P450 may be associated with the hypoxia-induced increase in ORS in the hepatocyte. The results of the in-vitro studies support the possibility that a source generating ORS can decrease cytochrome P450 activity. In addition, in the present study, the reduction in total amount of hepatic cytochrome P450 in hypoxic rabbits was inversely associated with the increase in lipid peroxidation ($r = 0.501$, $P < 0.05$) and with the intensity of microsomal chemiluminescence ($r = 0.584$, $P < 0.05$). Moreover, the specificity of the damaged isozymes support a suicide-type mechanism, i.e. liver tissues generate ORS that damage the nearest isozymes. Finally, it has been reported that pretreatment of rats with 10 000 int. units vitamin A reverses the effect of hypoxia on the activity of cytochrome P450, cytochrome c-reductase and aniline hydroxylase (Grover et al 1985); further studies are required to confirm that hypoxia-induced production of ORS is implicated in the reduction of total amount of cytochrome P450.

In summary, the present study demonstrates that in conscious rabbits acute normobaric moderate hypoxia for 24 h reduces the amount and activity of cytochrome P450 and promotes the presence of ORS. The apparently contradictory reports in the literature probably result from the differences in species, duration of exposure and severity of hypoxia. The present results support the hypothesis that in patients, acute hypoxia may result in a decrease in cytochrome activity, more specifically in the CYP 1A1 responsible for theophylline metabolism (Sarkar et al 1992).

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