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Effects of Chronic Normobaric Hypoxic and Hypercapnic Exposure in Rats: Prevention of Experimental Chronic Mountain Sickness by Hypercapnia

by

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ABSTRACT. — A syndrome of experimental chronic mountain sickness can be produced in the Hilltop strain of Sprague-Dawley rats by chronic hypobaric hypoxic exposure. This syndrome is characterized by polycythemia, plasma hemoglobinemia, pulmonary hypertension and right ventricular hypertrophy with eventual failure and death. It has generally been assumed that these changes are caused by chronic hypoxemia, not by hypobaric exposure *per se*. We have now confirmed this directly by showing that chronic normobaric hypoxic exposure (10.5% O_2) produces similar hematologic and hemodynamic changes. Further, the addition of hypercapnic exposure to the hypoxic exposure blunted or prevented the effects of the hypoxic exposure probably by stimulating respiration, thus increasing the rate of oxygen delivery to the cells. Changes in the rate-controlling enzymes of hepatic heme metabolism, 5-aminolevulinate synthase and heme oxygenase, and in cytochrome(s) P-450, the major hepatic hemoprotein(s), were also measured in hypoxic and hypercapnic rats. Hypoxia decreased 5-aminolevulinate synthase and increased cytochrome(s) P-450, probably by increasing the size of a "regulatory" heme pool within hepatocytes. These changes were also prevented by the addition of hypercapnic to hypoxic exposure.

INTRODUCTION

Hematological and cardiopulmonary responses to chronic hypoxia in the Hilltop strain of Sprague-Dawley rats have been studied extensively in our laboratory (Ou and Smith, 1978; Ou, 1980; Ou, Hill and Tenney, 1984; Hill and Ou, 1984). Of most significance is the syndrome of chronic mountain sickness caused by chronic hypoxia. This syndrome is characterized by severe polycythemia, pulmonary hypertension, right ventricular hypert-

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rophy and failure and eventual death (Ou and Smith, 1983, 1984; Ou, Hill and Tenney, 1984). Other unique hematological features in this strain of rat are the important contribution of splenic erythropoiesis to the polycythemia produced by chronic hypoxia (Ou et al., 1980), and the occurrence of hemoglobinemia (Ou and Smith, 1978; Ou, 1980).

The structure and function of organs other than the heart, lungs, spleen and bone marrow have not been studied extensively in rats with experimental chronic mountain sickness. However, in preliminary studies, the activities of renal and hepatic heme oxygenase (Ou and Smith, 1978), the rate-limiting enzyme of heme catabolism, and concentrations of hepatic cytochrome P-450 (Ou et al., 1980) were reported to be increased in such rats. The causative mechanism(s) for these and other changes produced by chronic hypobaric exposure have not been elucidated. Since hypoxia is a powerful physiological stimulus for both erythropoiesis (Erslev, 1977) and pulmonary hypertension (Fishman, 1976), it seems the likely cause for the hematological and cardiopulmonary changes observed during hypobaric exposure. If so, treatments that alleviate the severity of hypoxemia should eliminate or minimize these adverse pathological responses. In the present study, hypercapnia was used to test this hypothesis because hypercapnia is a physiological stimulant of breathing. In addition, hypercapnia is characteristically present in chronically hypoxemic patients suffering from heart, lung of blood diseases.

In this paper we report that chronic normobaric hypoxic exposure produces the same hematologic and hemodynamic changes as does chronic hypobaric exposure; furthermore, chronic hypercapnia diminishes or prevents these hypoxia-induced effects. Effects of hypoxia and hypercapnia on hepatic heme metabolism were also studied.

METHODS

Abbreviations: ALA, 5-aminolevulinate; BR, bilirubin; CoA, coenzyme A; LV, left ventricle; RV, right ventricle; S, septum of the heart.

RATS. Sprague-Dawly male rats, supplied by Hilltop Labs (Scottdale, PA), weighing 280-300 g, were used. Four groups of six animals each were exposed in the following conditions for 28-30 days: 1. Controls, exposed to normal room air (20.9% O_2); 2. room air plus CO_2 (6.0 ± 0.2%); 3. hypoxia (10.5% O_2); and 4. hypoxia plus CO_2 (10.5% O_2 + 6.0% CO_2). The rats were housed three per cage and the cages placed in sealed aquarium tanks (3x2x1.5 ft.). The sealed tanks were flushed with the appropriate gas mixtures, adjusted so that the O_2 and CO_2 contents of the out-flow gas were as desired. A sufficient amount of Drierite (Hammond Drierite Co., Xenia, OH) was used to absorb water and prevent water condensation. The tanks were opened every other day to replenish food and drinking water and to change cages. Rats were not starved prior to sacrifice. At the end of the exposure, animals were anesthetized with ether and exsanguinated via the vena cava, using an 18ga needle attached to a heparinized plastic disposable syringe.

TISSUE PREPARATIONS. Blood was carefully expressed from the syringes (without needles) into tubes containing EDTA. Microhematocrit tubes were filled, and the large tubes were then centrifuged (1,000xg, 15 min, 4°C) and plasma removed, care being taken to avoid the sedimented erythrocytes.

Livers were immediately perfused through the portal vein with 50-100 ml ice cold 0.15M NaCl. The blanched livers were then excised and weighed. A portion (~ 1 g) was used to prepare a 20% (w/v) homogenate in 0.25M sucrose/50mM Tris-HCl, pH 7.4/0.5mM

EDTA/0.2mM pyridoxal-5'-phosphate; this homogenate was prepared with a Potter-Elvehjem apparatus, using 10 up-and-down strokes of a motor-driven teflon pestle (600 rpm).

Another portion of each liver (~ 3 g) was homogenized with Potter-Elvehjem apparatus in 3 volumes of ice-cold 0.25M sucrose/0.02M Tris-Cl, pH 7.4. The homogenate was centrifuged (10,000 g, 10 min, 4°C). The supernatant fraction was removed and centrifuged (105,000 g, 45 min, 4°C). The pellet was washed twice and resuspended in 0.15M KCl, 0.01M Tris-Cl, pH 7.4 and recentrifuged (105,000 g, 50 min, 4°C). The pellet was again washed twice and resuspended in 0.1M KPO_4, pH 7.4/1mM EDTA/20% (w/v) glycerol. This suspension of microsomes could be frozen (–60°C) for at least 3 weaks without loss of cytochrome P-450 or activity of heme oxygenase.

After livers had been removed, the hearts were removed and weighed. They were then carefully separated into right ventricle (RV) and left ventricle (LV) and septum (S), as previously described (Fulton, Hutchinson and Jones, 1952).

ASSAYS. — Hematrocrits were measured with a standard microhematocrit reader (Damon-IEC microcapillary reader, Damon-IEC Co., Needham Hts., MA) after the capillary tubes had been centrifuged 5 min in a Damon-IEC MB microhematocrit centrifuge.

The pyridine hemochromogen method (Falk, 1964) was used to measure plasma heme concentrations. 0.05-0.1 ml of plasma was used for each determination. The concentration of plasma hemoglobin was then calculated assuming M_r of hemoglobin = 68,000 and 4 mols heme/mol hemoglobin.

Activities of ALA synthase were measured on freshly prepared liver homogenate, as previously described (Bonkowsky, Pomeroy and Sinclair, 1982; Lien and Beattie, 1982). Concentrations of microsomal cytochrome P-450 were measured by the method of Omura and Sato (1964). Activities of microsomal heme oxygenase were measured by a modification of previously described methods (Tenhunen, Marver and Schmid, 1969; Sunderman et al., 1982). The method used will be described in detail elsewhere (Lincoln et al., Manuscript in preparation).

Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

STATISTICS. — Data are presented as means ± SD. Significance of differences among groups was tested by analysis of variance procedures and by the procedure of Scheffe, using the SPSS statistical package of the Central Computer Facility of Dartmouth College. P values ≤ 0.05 were considered significant; p values between 0.06-0.10 were considered probably significant.

RESULTS AND DISCUSSION

EFFECTS OF HYPOXIA AND HYPERCAPNIA ON HEMATOLOGIC AND HEMODYNAMIC PARAMETERS. — As shown in Table 1, chronic normobaric hypoxia produced marked polycythemia and moderate hemoglobinemia. In general, these changes are similar to those observed previously in rats rendered chronically hypoxic by exposure to hypobaric conditions (Ou and Smith, 1978; Ou, Hill and Tenney, 1984), although the degree of increase was rather less in the current study. It seems evident that hypoxia, not

Table 1. Effects of chronic hypoxia and hypercapnia on hematocrits and plasma hemoglobin concentrations of rats.

Treatment	Hematocrit %	Plasma Hemoglobin Concentration (mg/dl)
Control (room air)	43 ± 1	15 ± 10
	(3)	(5)
Hypoxia (10.5% O_2)	71 ± 8^a	70 ± 40^a
	(3)	(5)
Hypercapnia (6% CO_2)	43 ± 1^b	10 ± 3^b
	(3)	(6)
Hypoxia + Hypercapnia	54 ± 1^b	19 ± 16^b
(10.4% O_2 = 6% CO_2)	(3)	(5)

Rats were exposed to various gas mixtures for 28-30d following which hematocrits and plasma hemoglobin concentrations were determined, as described in Methods. Results are mean ± 1 SD; Number of rats used are in parentheses.
[a] differs from control, $p < 0.05$; [b]differs from hypoxia, $p < 0.05$.

hypobaria *per se*, is responsible for the hematologic changes observed. Chronic hypercapnia alone had no effect on blood hematocrit or plasma hemoglobin concentration; however, the additon of hypercapnia to hypoxia prevented entirely the polycythemia and plasma hemoglobin increase produced by hypoxia alone.

Chronic normobaric hypoxia also produced cardiac hypertrophy (Fig. 1). The cardiac hypertrophy resulted solely from right ventricular hypertrophy (expressed either as right ventricular to body weight ratio (Fig. 1a) or as the ratio of left ventricular plus septal to right ventricular weight (Fig. 1c). There was no left ventricular hypertrophy (Fig. 1b). The present observations thus extend previous observations made in rats rendered chronically hypoxic by hypobaric exposure (Ou and Smith, 1983). Hypercapnia significantly suppressed the right ventricular cardiac hypertrophy produced by chronic hypoxia but had no cardiac effect under normal room air conditions.

In addition, chronic normobaric hypoxia resulted in enlargement of the spleen (Fig. 2), another result consistent with those obtained previously after chronic hypobaric hypoxic exposure (Ou, Kim et al., 1980). Hypercapnia prevented the hypoxia-induced splenomegaly.

Thus all the signs of chronic mountain sickness – severe polycythemia, pulmonary hypertension, right ventricular hypertrophy and eventual failure – previously observed in the Hilltop strain of Sprague-Dawley rats chronically exposed to hypobaric conditions (Heath and Williams, 1981; Ou and Smith, 1983; Ou, Hill and Tenney, 1984), were recapitulated by normobaric hypoxic exposure. These effects have been thought to be due to hypoxemia, resulting from relative hypoventilation (Heath and Williams, 1981). The present experiments support this hypothesis since the potent respiratory stimulus of hypercapnia blunted or prevented the signs of chronic mountain sickness. There is no reason to doubt that pulmonary vasoconstriction, produced by hypoxemia, contributes to the development of pulmonary hypertension, right ventricular hypertrophy and failure in chronic mountain sickness. Even though hypercapnia, through its acidotic

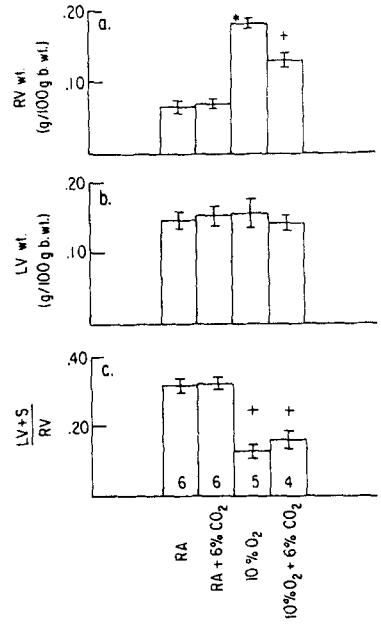

Fig. 1. Effects of chronic normobaric hypoxia and hypercapnia on cardiac ventricles. Mean values are plotted; error bars are ± S.D.; Numbers within the bars denote numbers of rats studied
*, Significantly different from each of the other groups; +, significantly different from control or from room air plus 6% CO_2.

Abbreviations: b.wt., body weight; LV, left ventricle; RA, room air; RV, right ventricle; S, septum

effect, potentiates the pulmonary vasoconstrictive response to hypoxemia (Fishman, 1976), in the present experiments hypercapnia minimized pulmonary hypertension, as shown by the lesser degree of right ventricular hypertrophy. Thus, the predominant effect of hypercapnia in our model is to stimulate breathing and decrease hypoxemia, rather than to potentiate pulmonary vasoconstriction which would lead to more severe pulmonary hypertension. A decrease in hypoxemia caused by hypercapnia probably accounts for all the effects produced in rats exposed to both hypoxia and hypercapnia.

EFFECTS OF HYPOXIA AND HYPERCAPNIA ON HEPATIC HEME METABOLISM AND CYTOCHROME P-450. — In a previous paper (Ou et al., 1980) we reported that chronic hypobaric exposure produced an increase in hepatic concentrations of cytochrome(s) P-450. This family of hemoproteins is quantitatively the major group of hepatic proteins for which heme is required (Bonkowsky et al., 1979; Bonkowsky, 1982) and is responsible for the initial (phase I) metabolism of many endogenous and exogenous chemicals, including steroids, cholesterol derivatives, drugs, toxins and carcinogens (Sato and Omura, 1979). Other aspects of heme metabolism in chronically

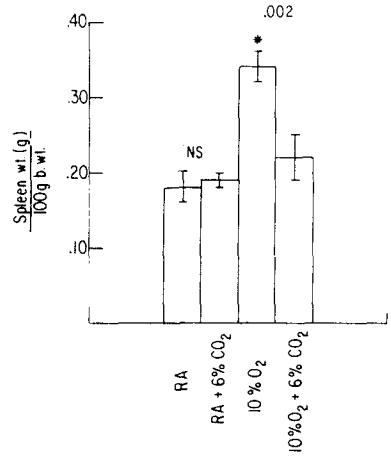

Fig. 2 Effects of chronic normobaric hypoxia and hypercapnia on spleen weight. Mean values are plotted; error bars show ± S.D.; numbers within bars denote numbers of rats studied.
* Significantly different from each of the other groups.

hypoxic animals had received little attention, although increases in hepatic and renal heme oxygenase had been found several years ago in hemoglobinemic, chronically hypoxemic rats (Ou and Smith, 1978). In rats and other mammals, heme oxygenase is the first and rate-limiting enzyme for heme breakdown (Tenhunen, Marver & Schmid, 1969; Kikuchi & Yoshida, 1983).

The rate-limiting enzyme of hepatic heme synthesis is ALA synthase, which catalyzes the first reaction in the pathway, namely, the condensation of glycine and succinyl-CoA to form ALA (Bonkowsky, 1982).

The normal or increased concentration of cytochrome P-450 and the lack of an increase in heme oxygenase also imply that hepatic functions and health were well maintained, despite chronic hypoxia. This conclusion is justified since in the past several years, studies have shown that numerous hepatotoxins produce decreased concentrations of hepatic cytochrome P-450 and increased activities in hepatic heme oxygenase. Examples of such toxins include heavy metals (Maines and Kappas, 1977; Kikuchi and Yoshida, 1983), endotoxin (Bissell and Hammaker, 1976), allyl alcohol, bromobenzene, furosemide, diethylmaleate (Guzelian and Elshourbagy, 1979; Kikuchi and Yoshida, 1983), arsenicals (Sardana et al., 1981), chlorinated benzenes (Ariyoshi et al., 1981), ethionine (Matsuura et al., 1984) and thioacetamide and its S-oxide metabolite (Matsuura et al., 1983). Despite numerous studies, the mechanism for induction of the oxygenase by such treatments remains unclear. Regardless of mechanism, however, it seems that, in addition to the substrate-mediated induction of heme oxygenase produced by heme (Bonkowsky, 1982; Kikuchi and Yoshida, 1983), activity of this enzyme is increased by hepatotoxic conditions. The fact that, in our studies, hypoxic and hypercapnic exposures increased concentrations of cytochrome P-450 without affecting heme oxygenase, indicates that such exposures do not in themselves cause hepatotoxicity.

We are uncertain why, in the current study, we failed to observe increased hepatic

Table 2. Effects of chronic hypoxia and hypercapnia on ALA synthase, heme oxygenase and cytoebrome PH50 in rats.

Treatment	Homogenate ALA Synthase (nmol ALA/mg protein per h)	Microsoma 1 Heme Oxygenase (nmol BR/mg protein per h)	Microsoma 1 Cytochrome P-450 (nmol/mg protein)
Control (room air)	0.51 ± 0.21 (6)	5.9 ± 1.1 (6)	1.29 ± 0.04 (6)
Hypoxia (10.5% O_2)	0.23 ± 0.05[a] (4)	7.6 ± 1.7 (4)	1.44 ± 0.14[c] (4)
Hypercapnia (6% CO_2)	0.41 ± 0.17[b] (6)	7.0 ± 0.66 (5)	1.45 ± 0.12[c] (6)
Hypoxia + Hypercapnia (10.4% O_2 = 6% CO_2)	0.53 ± 0.18[b] (5)	7.3 ± 1.6 (4)	1.21 ± 0.18[d] (5)

Rats were exposed to various gas mixtures for 28-30d, following which activities of hepatic enzymes were measured as described in Methods. Values are m ± SD; Numbers of rats used in parentheses.

[a] differs from control, $p < 0.05$
[b] differs from hypoxia, $p < 0.05$
[c] probably differs from control, $p < 0.10$
[d] probably differs from hypercapnia, $p < 0.10$

Abbreviations: ALA, 5-aminolevulinate; BR, bilirubin

heme oxygenase activity in chronically hypoxic rats, whereas, in an earlier study (Ou and Smith, 1978) increased activity was observed. Somewhat different assay procedures were used in the two experiments: the duration of hypoxic exposure was longer in the earlier than in the current study (40d vs 30d); and the degree of the hemoglobinemia was probably greater previously since all rats had gross hemoglobinuria, a condition not observed in the rats studied here. In the earlier study activities of heme oxygenase in the livers of control rats were unusually low (ca. 0.1 nmol BR/mg protein per h). Thus, even after the 5-fold increase in activity produced by hypoxic exposure, the activities were still less than the activities we observed here (Table 2) or that others generally report in control rats (Tenhunen, Marver and Schmid, 1969; Maines and Kappas, 1977; Guzelian and Elsbourbagy, 1979; Sardana et al., 1981). To be certain that our current assay was capable of detecting increases in heme oxygenase activity, had they been present, we confirmed that administration of $CoCl_2$ (250 μmol/kg body wt), an agent well-known to induce heme oxygenase (Maines and Kappas, 1977; Kikuchi and Yoshida, 1983), produced a marked increase in activity of the enzyme in rats (data not shown).

In the present study we measured activities of hepatic ALA synthase and heme oxygenase, as well as concentrations of cytochrome P-450 in control, hypoxic and hypercapnic rats. As shown in Table 2, chronic hypoxia significantly decreased activities of ALA synthase and increased concentrations of cytochrome P-450. However, the 29% increase in heme oxygenase produced by chronic hypoxia did not achieve statistical significance perhaps because of rather large variances in the data. The changes in ALA

synthase and cytochrome P-450 produced by chronic hypoxia were prevented by concomitant exposure to hypercapnia. These results, taken with those for hematocrit and plasma free hemoglobin concentrations discussed above, suggest that, in the hypoxic rats, the "regulatory" pool of hepatocyte heme (Bonkowsky, Sinclair and Sinclair, 1979; Bonkowsky, 1982) is increased slightly, sufficient to repress activity of ALA synthase, but insufficient to induce heme oxygenase activity. It seems likely that an increase in the size of the regulatory heme pool is related to the increase in plasma hemoglobin concentrations observed in hypoxic rats.

Our failure to observe a large increase in heme oxygenase activity in the hypoxic rats indicates that, in our rats, the increase in size of the hepatic "regulatory" heme pool was relatively small. Because of different sensitivities to heme, the process of repression of ALA synthase occurs at lower heme concentrations than the process of induction of heme oxygenase (Granick et al., 1975). The decrease in activity of ALA synthase in hypoxic rats cannot be due to a starvation effect in hypoxic rats, since fasting increases, whereas feeding decreases, activity of this enzyme (Bonkowsky et al., 1979, 1982).

One or more of the following may contribute to the increase in concentration of cytochrome P-450 produced by chronic hypoxia: 1. An increase in the "regulatory" heme pool size may supply additional heme to combine with the protein moiety of cytochrome P-450 to produce holo-cytochrome P-450; 2. The rate of breakdown of cytochrome P-450 caused by "active" oxygen radicals or other oxidizing free radicals may be decreased in chronic hypoxia. There is evidence that such radicals can degrade cytochrome P-450 (Hrycay and O'Brien, 1971; Schacter, Marver and Meyer, 1972), and it is reasonable to speculate that such species are decreased in livers of chronically hypoxic rats; 3. The rate of synthesis of cytochrome(s) P-450 may be increased in response to lowered hepacytic pO_2 values. Perhaps concentrations of this critical family of O_2-requiring hemoproteins are regulated in part by O_2 supply.

Other possible explanations for the different responses of heme oxygenase observed in the two experiments are 1. that genetic drift in the Hilltop strain of Sprague-Dawley rats occurred between 1977 and 1984, or 2. that there was a difference due to the fact that the hypoxia was here produced under normobaric, rather than hypobaric conditions.

In summary, the blunting of hematological, metabolic and cardiopulmonary responses to chronic hypoxia by hypercapnic exposure can be attributed to further stimulation of the ventilatory response to hypoxia by hypercapnia. Our results justify use of chronic hypobaric exposure to produce chronic hypoxemia: hypobaric exposure produces changes of chronic mountain sickness equivalent to those produced by chronic normobaric hypoxic exposure and does so with much less expense, since costly gas mixtures need not be used.

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