Effects of hypobaric hypoxia on antioxidant enzymes in rats

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1. The present study was undertaken to investigate the effects of hypobaric hypoxia, equivalent to an altitude of 5500 m, on antioxidant enzymes in rats.

2. Malondialdehyde levels in serum, heart, lung, liver and kidney of hypobaric-hypoxic rats were all significantly higher than in control rats by day 21 of exposure ($P < 0.05$), indicating increased oxidative stress.

3. Superoxide dismutase (SOD) catalyses the conversion of the superoxide anion to H_2O_2 and O_2. The concentration of immunoreactive Mn-SOD in the serum of hypobaric-hypoxic rats was raised significantly from day 5 onwards, whereas in liver and lung, it had decreased significantly by day 21 ($P < 0.05$).

4. Glutathione peroxidase (GSH-Px) catalyses H_2O_2 and certain lipid peroxides. By day 21, GSH-Px activity had increased significantly in the heart and lungs, but decreased significantly in the liver ($P < 0.05$).

5. Catalase catalyses H_2O_2. Catalase activity in the liver and kidney of hypobaric-hypoxic rats was significantly decreased on day 1 ($P < 0.05$) though levels then recovered.

6. Mn-SOD mRNA in the liver of hypobaric-hypoxic rats was induced during the experiment, the effect being exceptionally marked, especially during the first 3 days of exposure to hypobaric hypoxia.

7. These results suggest that the liver may be more vulnerable than the other organs tested to oxidative stress under hypobaric hypoxia.

Oxidative stress is an important cause of in-cell damage. Consequently, numerous studies have been published on the effects on the antioxidant systems of conditions in which the level of tissue oxygenation is changed such as: (i) hypoxia (Liu, Simon, Phillips & Robin, 1977; Yoshikawa, Furukawa, Wakamatsu, Takemura, Tanaka & Kondo, 1982; Bonkovsky, Lincoln, Healey, Ou, Sinclair & Muller-Eberhard, 1986; De Groot & Littauer, 1989; Costa, 1990; Dhaliwal, Kirshenbaum, Randhawa & Singal, 1991; Simon-Schnass, 1992); (ii) physical training (Ohno *et al.* 1986; Ohno, Yahata, Sato, Yamamura & Taniguchi, 1988; Ohno, Kayashima, Nagata, Yamashita, Ookawara & Taniguchi, 1993); (iii) aging (Taniguchi, 1992), and (iv) cancer (Taniguchi, 1992). In general, the formation of oxygen-derived free radicals decreases during hypoxia (De Groot & Littauer, 1989). However, prolonged exposure to high altitude (5100 m) has been reported to cause a marked increase in the level of expired pentane. This increase suggests an increase in lipid peroxidation (Simon-Schnass, 1992), which may indicate increased oxidative stress. Even though antioxidant systems have been the subject of considerable research, little is known of the changes, if any, in the levels of antioxidant activity that occur in hypobaric hypoxia.

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) protect tissues from oxidative stress (Ohno *et al.* 1986, 1988, 1993; Taniguchi, 1992). SOD catalyses the conversion of the superoxide anion (O_2^-), which is the first product of O_2 radicals, to H_2O_2 and O_2. Mammalian tissues contain two primary forms of SOD: Mn-SOD, which is found predominantly in the mitochondrial matrix (where there is likely to be an elevated rate of O_2 utilization), and CuZn-SOD, which is

mainly present in the cytoplasm. Both GSH-Px and CAT break down H_2O_2 and, in addition, GSH-Px catalyses the reduction of certain lipid peroxides. In the present study, we investigated the effects of a level of hypobaric hypoxia equivalent to an altitude of 5500 m on antioxidant enzymes in rats. We measured malondialdehyde (MDA) activity as an index of lipid peroxidation, the level of Mn-SOD, the expression of Mn-SOD mRNA and the activities of GSH-Px and CAT. In so doing, we hoped to contribute to the understanding of the role of antioxidant enzymes in the response to oxidative stress at high altitude.

METHODS

Exposure of animals to hypobaric hypoxia

Forty male Wistar rats (8 weeks old) weighing 250–350 g were divided into eight groups of five animals each. Each group was housed in an experimental hypobaric-hypoxic chamber and exposed to conditions equivalent to those found at 5500 m (0·5 ATA (atmospheres absolute), 380 mmHg = 60·5 kPa; F_{I,O_2} (fractional inspired oxygen), 0·105) for a period of 12 h or 1, 3, 5, 7, 14 or 21 days. At the end of their exposure period, the rats were decapitated and the serum, liver, lungs, heart, kidneys and gastrocnemius muscle removed. The samples were immediately frozen in liquid nitrogen and stored at −80 °C. Prior to the experiments, the rats had been reared at an ambient temperature of 23 ± 1 °C and a relative humidity of 60–70%, with light exposure daily from 6.00 to 18.00 h. One group of five rats was kept in a sea-level-altitude environment and served as a control group. The data from this group are shown as that from '0 days hypobaric hypoxia' in the figures. Each animal received a standard laboratory diet and tap water *ad libitum* throughout the experimental period. The animals were cared for in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (no. 85-23, 1985), Bethesda, MD, USA.

Tissue preparations and assays

All tissue extracts were prepared by homogenization in four volumes of buffer containing 0·25 M sucrose, 0·1 mM ethylenediaminetetraacetic acid and tris(hydroxymethyl)aminomethane chloride (pH 7·4) in a Potter–Elvehjem homogenizer, followed by centrifugation (700 *g* for 10 min). Each supernatant was then sonicated and stored at −80 °C. MDA content was determined using the thiobarbituric acid method (Ohkawa, Ohishi & Yagi, 1979).

Immunoreactive Mn-SOD was measured using an enzyme-linked immunosorbent assay with a polyclonal antibody raised against purified Mn-SOD from rat liver (Suzuki, Nakata, Seo, Miyazawa, Sugiyama & Taniguchi, 1991). We chose to measure the level of immunoreactive Mn-SOD because activity measurements alone may not reflect the actual amount of Mn-SOD protein present: the activity depends on the presence or absence of cofactors, activators and inhibitors (Taniguchi, 1992). Fortunately, the Mn-SOD level in serum and tissues is not affected by haemolysis, unlike that of CuZn-SOD (Taniguchi, 1992). Briefly, 50 μl of the polyclonal antibody was added to each well of microtitre plates and the plates were then incubated at 4 °C for 2 h. Each well was then washed, filled with 0·1% (w/v) bovine serum albumin and incubated at 4 °C for 2 h. The wells were washed again to remove unbound protein and 50 μl of diluted supernatant derived from one of the tissue samples was added to each antibody-coated well. After incubation for 2 h at room temperature, the wells were washed to remove unbound antigen and biotin-conjugated antibody was added to each well. Following incubation for another 2 h at room temperature, the unbound antibody was removed by an additional wash. Avidin-conjugated horseradish peroxidase (HRP) was then added to each well and incubated for 10 min at room temperature. The wells were washed and the substrate for HRP (0·05% H_2O_2 in 0·1 M sodium citrate buffer, containing 0·6 mg ml^{-1} *o*-phenylenediamine; pH 5·4) added to each well. After a 10 min incubation at room temperature, 1 N H_2SO_4 was added to stop the enzymatic reaction and the absorbance measured at 492 nm using a microplate reader.

The activity of GSH-Px was determined by continuous monitoring of the formation of oxidized glutathione at 340 nm (Chance & Maehly, 1955). To measure CAT activity, the level of O_2 formation was continuously monitored at 230 nm (Tappel, 1978).

Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). In addition, the activities in the serum of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with an autoanalyser, using commercially available activity kits (Daiichi Pure Chemicals, Tokyo). The AST and ALT activities were used as markers for monitoring liver function.

The expression of Mn-SOD mRNA in the liver of three rats from each group was examined using Northern blot analysis (Maniatis, Fritsch & Sambrook, 1982). Total liver RNA was isolated using acid guanidinium isothiocyanate-phenol-chloroform extraction and ethanol precipitation (Chomczynski & Sacchi, 1987). Following extraction, 30 μg of the total RNA was isolated by electrophoresis on a 1·5% agarose gel containing 8% formaldehyde and then transferred to a cellulose membrane by capillary action. A portion of rat Mn-SOD cDNA, containing 440 bp in the region of 139 to 578, was used as a probe, with β-actin cDNA being used as an internal control. The membrane was hybridized with a ^{32}P-labelled Mn-SOD cDNA probe for 16 h at 42 °C, washed in 2 × SSC and 1 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate) containing 0·1% sodium dodecyl sulphate for 20 min and then exposed to Amersham Hyperfilm at −80 °C for up to 8 days. To obtain an independent measurement of the integrity and amount of RNA on the membrane, the same blots were washed and rehybridized using a β-actin cDNA probe.

Statistical analysis

The results are expressed as the mean ± S.E.M. Fisher's protected least-significant-difference test was applied to the data when significant F ratios were obtained using analysis of variance (ANOVA). All differences stated to be 'significant' were at the level $P < 0·05$.

RESULTS

Body weight

Rats in the sea-level control group had a significantly higher body weight than those in the group exposed to hypobaric hypoxia for 21 days (329 ± 6 *vs.* 292 ± 4 g; Fig. 1).

Lipid peroxidation

The MDA level in the serum of rats exposed to hypobaric hypoxia for 14 or 21 days was significantly higher than that

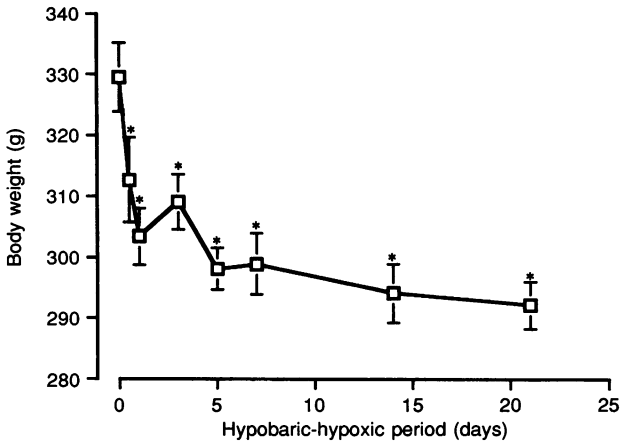

Figure 1

Body weight under hypobaric hypoxia. * Significantly different from control group ($P < 0.05$).

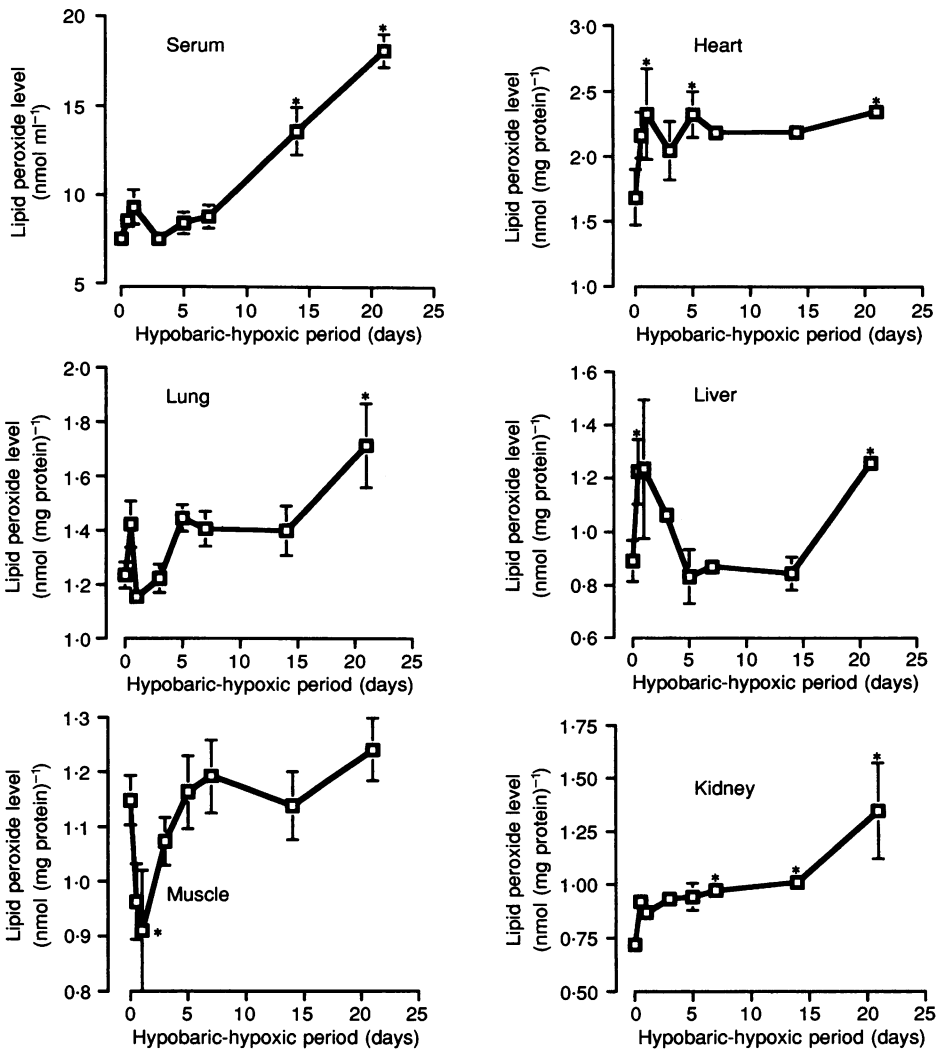

Figure 2

The concentration of lipid peroxides, expressed as the level of malondialdehyde, in serum and major organs under hypobaric hypoxia. * Significantly different from control group ($P < 0.05$). Here and in subsequent figures, where error bars are not shown they have been obscured by symbol.

of the controls (Fig. 2). Moreover, the MDA levels in the heart, lung, liver and kidney tissues of hypobaric-hypoxic rats in the 21 day group were significantly higher than the corresponding levels in the control group. A biphasic elevation of MDA was noted in the liver: an early elevation occurred after a 12 h exposure, with an additional elevation occurring after 21 days exposure. In contrast to the other tissues, the gastrocnemius muscle of the hypobaric-hypoxic rats in the 12 h group showed an MDA level that was significantly lower than that in the control group.

Antioxidant enzymes

The concentration of immunoreactive Mn-SOD in the serum of hypobaric-hypoxic rats was significantly higher than that of the controls from day 5 to day 21 (Fig. 3). The level of Mn-SOD in control heart tissue was 981 ± 90 ng (mg protein)$^{-1}$, considerably higher than the level in the other organs, and that in the heart of hypobaric-hypoxic rats was significantly higher than control on day 1, but not thereafter. By contrast, the Mn-SOD concentrations in the liver and lungs were significantly lower than control throughout the experiment, with some exceptions: liver levels from 12 h to day 5 and lung levels on days 5 and 14. No significant difference in Mn-SOD levels in the kidney or gastrocnemius muscle was observed between control and experimental groups.

The activity of GSH-Px in the heart after 1, 14 and 21 days of exposure to hypobaric hypoxia was significantly higher than in the controls, as it was in the lungs at 21 days (Fig. 4). In contrast, GSH-Px activity in the liver was significantly lower than in the controls on day 21. No

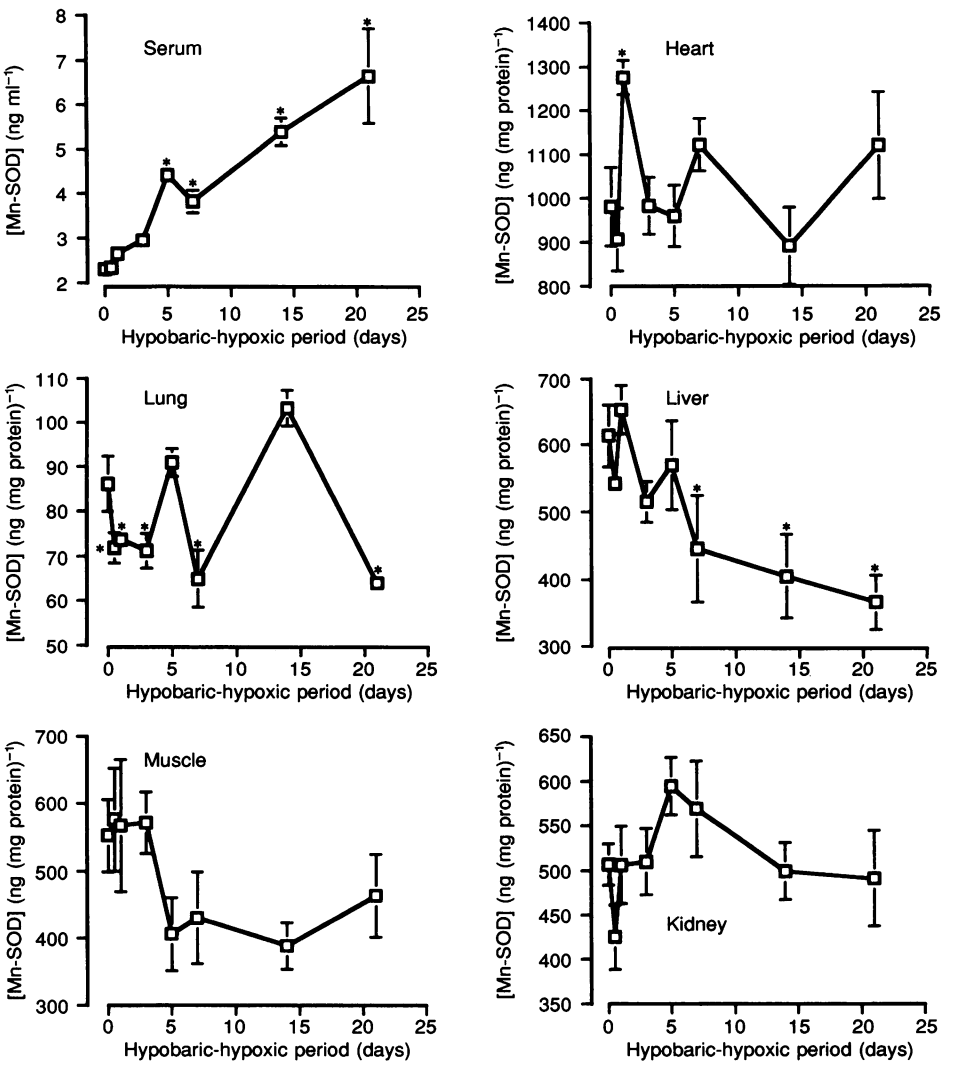

Figure 3

The concentration of Mn-SOD in serum and major organs under hypobaric hypoxia. * Significantly different from control group ($P < 0{\cdot}05$).

significant change in GSH-Px activity was observed in the kidney or gastrocnemius muscle.

Liver CAT activity was significantly lower than in the controls at 12 h and on days 1 and 5. Kidney CAT activity was significantly lower on day 1 (Fig. 5). In contrast, CAT activities in the heart, lungs and gastrocnemius muscle were not significantly affected by exposure to hypobaric hypoxia.

Expression of Mn-SOD mRNA

Mn-SOD mRNA in the liver of control rats was present on the gel as two major bands of 4 and 1 kb (Fig. 6). Mn-SOD mRNA in the liver of hypobaric-hypoxic rats was markedly increased, especially during the first 3 days of exposure to hypobaric hypoxia.

AST and ALT in sera

The activities of AST and ALT in the serum were not significantly affected by exposure to hypobaric hypoxia (data not shown).

DISCUSSION

Although several reports have been published on the antioxidant system during conditions of hypobaric hypoxia (Liu *et al.* 1977; Bonkovsky *et al.* 1986; Costa, 1990), none have focused specifically on the effects on antioxidant enzymes of hypobaric hypoxia. In the present study, we examined the levels of Mn-SOD and other antioxidant enzymes in the serum and major organs and the expression of Mn-SOD mRNA in the liver of rats during exposure to

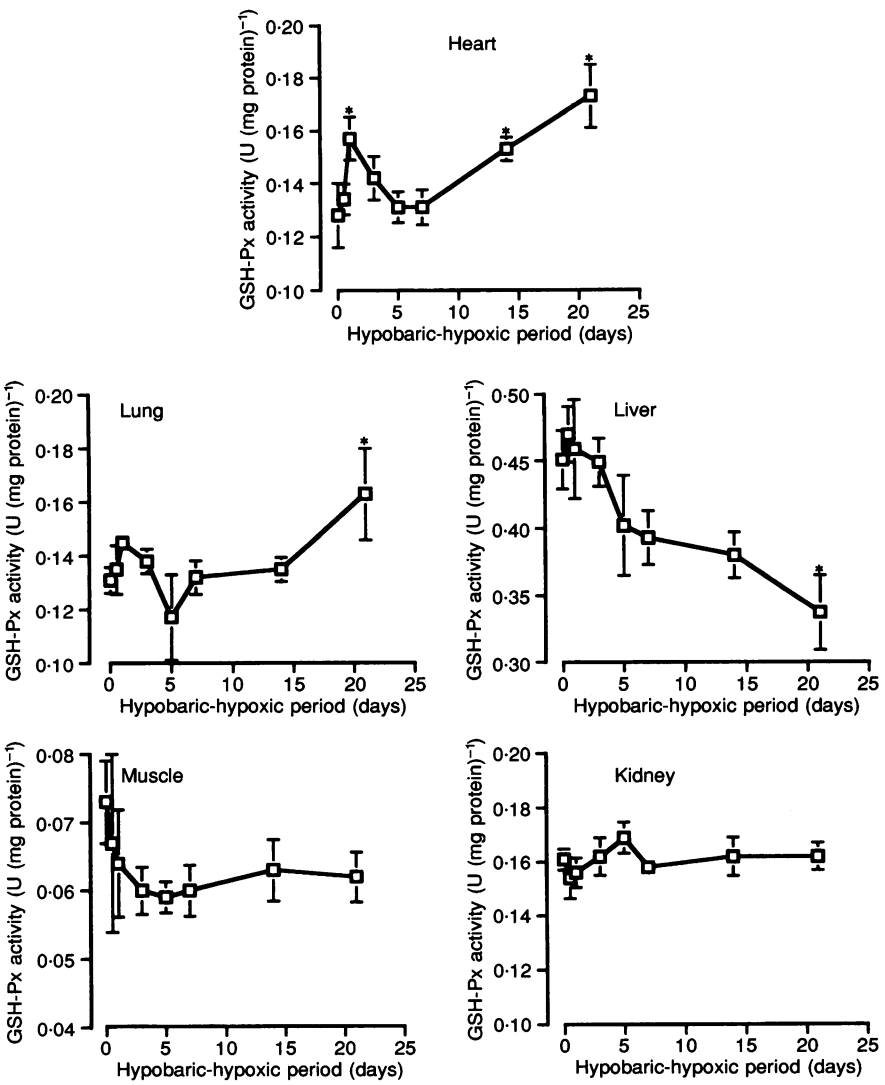

Figure 4

The activity of glutathione peroxidase (GSH-Px) in major organs under hypobaric hypoxia. * Significantly different from control group ($P < 0.05$).

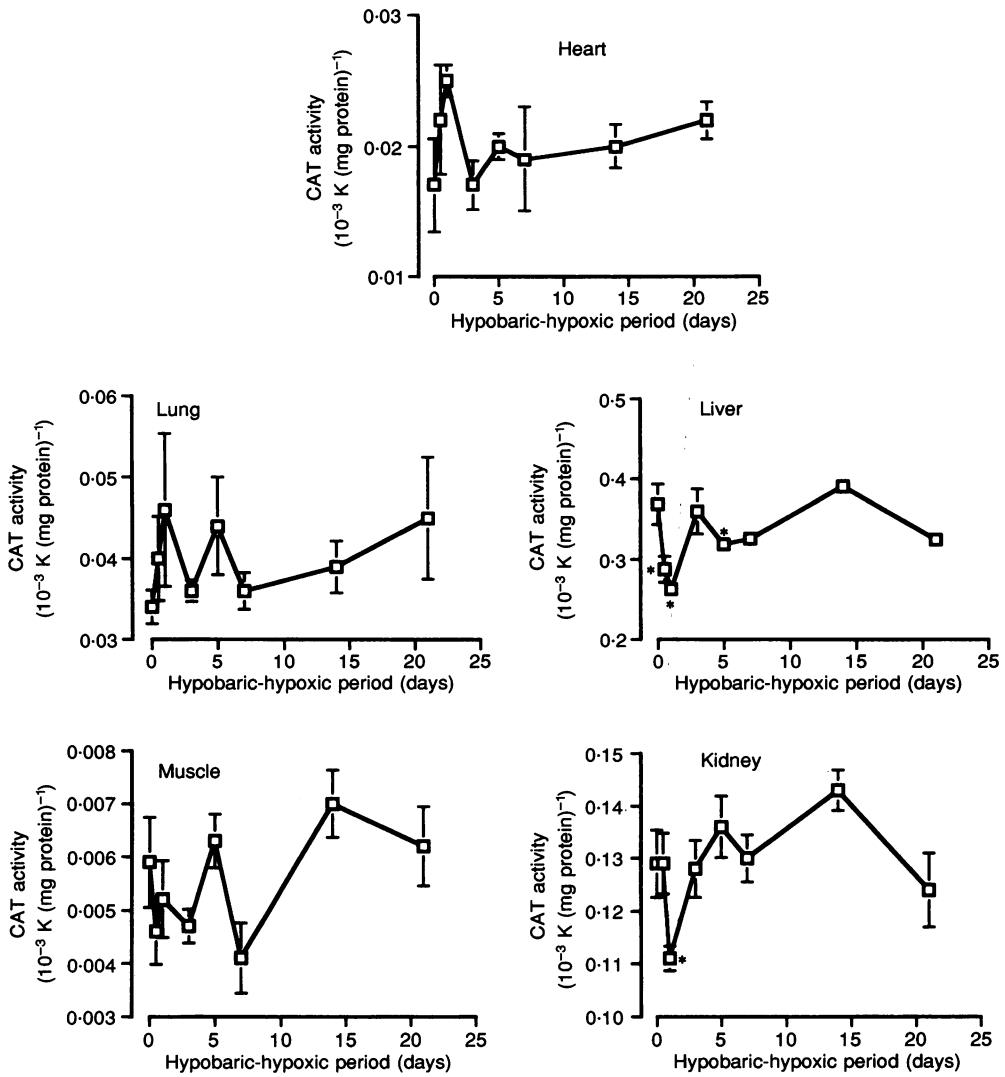

Figure 5

The activity of CAT (K) in major organs under hypobaric hypoxia; $K = 2{\cdot}3/dt(\log X_1/X_2)$, where dt is change in time, X_1 is initial absorption and X_2 is final absorption. * Significantly different from control group ($P < 0{\cdot}05$).

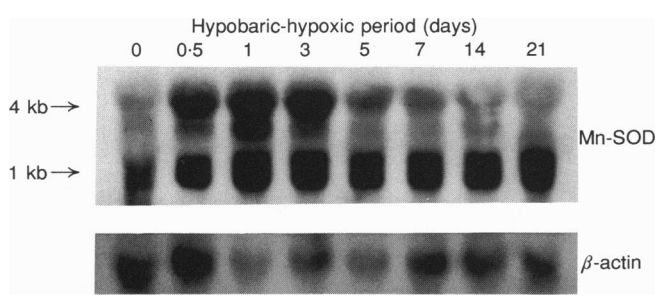

Figure 6

The expression of Mn-SOD mRNA in the liver under a hypobaric-hypoxic environment, as detected by Northern blotting and hybridization using β-actin, a rat Mn-SOD cDNA probe.

hypobaric hypoxia. We also observed over the same period the level of MDA, which gives an indication of the end-products of lipid peroxidation.

In the serum, heart, lungs, kidneys and liver, the level of MDA had increased by the 21st day of exposure to hypobaric hypoxia. This suggests an increase in the level of oxidative stress and is similar to results obtained by Yoshikawa *et al.* (1982). The oxidative stress seemed to increase with the length of the exposure to hypobaric hypoxia in that serum MDA levels tended to increase progressively, reaching maximum levels at the end of the experiment. However, during the initial 5 days of exposure, the influence of oxidative stress on the major organs was not uniform, the MDA level increasing significantly in the heart and liver, but decreasing in the gastrocnemius muscle. There was also a marked decrease (>10%) in body weight during the 21 days of exposure to hypobaric hypoxia. Similarly, a loss of body weight was seen in rats exposed to hypobaric hypoxia of 0·5 ATA for 2 weeks or 52 days (Meyrick & Reid, 1978, 1979). It may be partly attributable to anorexia or to an increased metabolic rate produced by the hypobaric-hypoxic stress (Meyrick & Reid, 1978, 1979; Simon-Schnass, 1992), but it could also have been caused by the oxidative stress itself (Simon-Schnass, 1992).

Mn-SOD has been implicated in aging and disease and high levels of serum Mn-SOD are present in patients with certain malignancies and in those with an acute myocardial infarction (Taniguchi, 1992). Although there may be conflicting influences on Mn-SOD in a physiological situation such as exercise, we have previously reported that increases in the serum level of Mn-SOD occurred in human subjects during an 8-day recovery period after 93 h of strenuous physical exercise. This increase resulted mainly from skeletal muscle damage during the early phase and liver damage during the later phase (Ohno *et al.* 1993). In the present study, the Mn-SOD level in the serum increased gradually with the increase in exposure to hypobaric hypoxia, reaching its highest value at 21 days. However, this increase in serum Mn-SOD level cannot easily be related to changes in the Mn-SOD levels of the major organs, because the Mn-SOD levels in the organs tested did not simply increase after day 5 of hypobaric hypoxia. In fact, this data showed considerable variability (especially in the lungs) for reasons that are unclear, except in the liver, where Mn-SOD levels fell, rather than rose, after day 5. It is highly unlikely that Mn-SOD leaked from liver cells into the bloodstream, because AST and ALT activities in the serum did not change significantly over our experimental period. Thus, the mechanism responsible for the increase in serum Mn-SOD levels is unclear.

We recently reported that endothelial cells are rich in Mn-SOD and that the enzyme in these cells is strongly induced during ischaemia by cytokines, such as tumor necrosis factor and interleukin-1 (Suzuki *et al.* 1992, 1993). This raises the possibility that the increase in serum Mn-SOD levels in hypobaric hypoxia may be partly the result of the release of Mn-SOD into the bloodstream from endothelial cells throughout the body. However, if this was the case, and since the lungs are so rich in endothelium, the level of Mn-SOD in the lungs would be expected to rise during hypobaric hypoxia. In fact, although our lung data showed considerable variability (Fig. 3), the only significant changes were in the downward, not the upward direction. Thus, our data do not permit any firm conclusion to be drawn on this point.

The present study also revealed significant decreases in Mn-SOD and in GSH-Px and CAT activities in the liver during exposure to hypobaric hypoxia. This suggests that the liver may be more vulnerable to oxidative stress than other organs during hypobaric hypoxia. It is particularly interesting that, even though the levels of Mn-SOD fell in the liver especially during the last 14 days, there were marked increases in the expression of Mn-SOD mRNA in this organ, especially in the first 3 days. It may be that the early elevation in the liver level of MDA (see above) stimulated the expression of Mn-SOD mRNA, whereas the increase in the expression of Mn-SOD mRNA during the last 14 days was the result of the decrease in the Mn-SOD level, via a negative-feedback system. As the discrepancy between the decreases in Mn-SOD levels and over-expression of Mn-SOD mRNA in the liver continued throughout the experiment, it would appear that the liver could not produce proteins from Mn-SOD mRNA, possibly because of impaired regulation during and after Mn-SOD translation.

Although there was no antioxidant enzyme response in the liver, we did find increased GSH-Px activity and a tendency towards increased CAT activity in the heart and the lungs during exposure to hypobaric hypoxia. These increases in antioxidant enzyme activities occurred roughly in parallel with the increased levels of lipid peroxidation in both organs. Therefore, these two organs would appear to show greater resistance to the oxidative stress produced by exposure to hypobaric hypoxia. Simon-Schnass (1992) suggested that antioxidants may act to offset the decrease in physiological performance that occurs during a prolonged stay at high altitude because they protect against oxidative injury mediated by free radicals. However, the present results appear inconsistent with this view inasmuch as lipid peroxidation increased, rather than decreased, in the heart, lungs, kidneys and liver during hypobaric hypoxia lasting 21 days. Moreover, the levels of Mn-SOD in the liver and lungs, the levels of GSH-Px in the liver, and the level of CAT in the liver and kidneys decreased, at least at some time points, during the hypobaric hypoxia. Clearly, more reseach is needed before a firm conclusion can be drawn.

Bonkovsky, H. L., Lincoln, B., Healey, J. F., Ou, L. C., Sinclair, P. R. & Muller-Eberhard, U. (1986). Hepatic heme and drug metabolism in rats with chronic mountain sickness. *American Journal of Physiology* **251**, G467–474.

Chance, B. & Maehly, A. C. (1955). Assay of catalases and peroxidases. *Methods in Enzymology* **2**, 764–775.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

Costa, L. E. (1990). Hepatic cytochrome P-450 in rats submitted to chronic hypobaric hypoxia. *American Journal of Physiology* **259**, C654–659.

De Groot, H. & Littauer, A. (1989). Hypoxia, reactive oxygen, and cell injury. *Free Radical Biology and Medicine* **6**, 541–551.

Dhaliwal, H., Kirshenbaum, L. A., Randhawa, A. K. & Singal, P. K. (1991). Correlation between antioxidant changes during hypoxia and recovery on reoxygenation. *American Journal of Physiology* **261**, H632–638.

Liu, J., Simon, L. M., Phillips, J. R. & Robin, E. D. (1977). Superoxide dismutase (SOD) activity in hypoxic mammalian systems. *Journal of Applied Physiology* **42**, 107–110.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*, pp. 7.39–7.52. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

Meyrick, B. & Reid, L. (1978). The effect of continued hypoxia on rat pulmonary arterial circulation: An ultrastructural study. *Laboratory Investigation* **38**, 188–200.

Meyrick, B. & Reid, L. (1979). Hypoxia and incorporation of ^{3}H-thymidine by cells of the rat pulmonary arteries and alveolar wall. *American Journal of Pathology* **96**, 51–70.

Ohkawa, H., Ohishi, N. & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* **95**, 351–358.

Ohno, H., Kayashima, S., Nagata, N., Yamashita, H., Ookawara, T. & Taniguchi, N. (1993). Changes in immunoreactive manganese-superoxide dismutase concentration in human serum after 93 h strenuous physical exercise. *Clinica Chimica Acta* **215**, 213–219.

Ohno, H., Sato, Y., Yamashita, K., Doi, R., Arai, K., Kondo, T. & Taniguchi, N. (1986). The effect of brief physical exercise on free radical scavenging enzyme systems in human red blood cells. *Canadian Journal of Physiology and Pharmacology* **64**, 1263–1265.

Ohno, H., Yahata, T., Sato, Y., Yamamura, K. & Taniguchi, N. (1988). Physical training and fasting erythrocyte activities of free radical scavenging enzyme systems in sedentary men. *European Journal of Applied Physiology* **57**, 173–176.

Simon-Schnass, I. M. (1992). Nutrition at high altitude. *Journal of Nutrition* **122**, 778–781.

Suzuki, K., Kinoshita, N., Matsuda, Y., Higashiyama, S., Kuzuya, T., Minamino, T., Tada, M. & Taniguchi, N. (1992). Elevation of immunoreactive serum Mn-superoxide dismutase in patients with acute myocardial infarction. *Free Radical Research Communications* **15**, 325–334.

Suzuki, K., Nakata, T., Seo, H. G., Miyazawa, N., Sugiyama, T. & Taniguchi, N. (1991). Differential expression of Mn- and Cu, Zn-superoxide dismutases in various tissues of LEC rats. In *The LEC Rats: A New Model for Hepatitis and Liver Cancer*, ed. Mori, M., Yoshida, M. C., Takeichi, N. & Taniguchi, N., pp. 142–148. Springer-Verlag, Tokyo.

Suzuki, K., Tatsumi, H., Satoh, S., Senda, T., Nakata, T., Fujii, J. & Taniguchi, N. (1993). Manganese-superoxide dismutase in endothelial cells: Localization and mechanism of induction. *American Journal of Physiology* **265**, H1173–1178.

Taniguchi, N. (1992). Clinical significances of superoxide dismutases: Changes in aging, diabetes, ischemia, and cancer. *Advances in Clinical Chemistry* **29**, 1–59.

Tappel, A. L. (1978). Glutathione peroxidase and hydroperoxides. *Methods in Enzymology* **52**, 506–513.

Yoshikawa, T., Furukawa, Y., Wakamatsu, Y., Takemura, S., Tanaka, H. & Kondo, M. (1982). Experimental hypoxia and lipid peroxide in rats. *Biochemical Medicine* **27**, 207–213.

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