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Enhancement of Rat Serum Ceruloplasmin Levels by Exposure to Hyperoxia (41917)

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Abstract. Exposure of rats to elevated oxygen tensions is a well-known method of producing enhanced levels of pulmonary antioxidant enzymes. Ceruloplasmin is a serum constituent which possesses scavenging activity toward oxygen radicals. Rats exposed either to continuous 85% oxygen for 7 days or to intermittent hyperbaric oxygen for up to 19 days developed not only enhanced lung antioxidant enzymes but also increased levels of serum ceruloplasmin. The latter did not appear to be merely an "acute phase reactant" as there was no change in total serum protein, plasma fibrinogen, serum -SH groups, sedimentation rate, or serum iron. Induction of ceruloplasmin may account for some of the anti-inflammatory activities of elevated oxygen tensions. © 1984 Society for Experimental Biology and Medicine.

Exposure of mammalian organisms to prolonged, elevated oxygen tensions generally produces substantial toxicity. For example, rats usually demonstrate 100% mortality after 72 hr in pure oxygen. In some circumstances, however, controlled exposure to oxygen can have beneficial and anti-inflammatory effects. In particular, intermittent hyperbaric oxygenation has been reported to ameliorate allergic encephalomyelitis in guinea pigs (1) or adjuvant arthritis in rats (2). It is also well known that various oxygen exposure regimens will induce elevated levels of lung radical-scavenging enzymes, especially superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (3–7).

We were intrigued by the observation that hyperbaric oxygenation could forestall or even abolish adjuvant arthritis (AA) in rats, since there has also been a report that administration of exogenous SOD provided similar beneficial effects on this widely used animal model of inflammatory disease (8). We, therefore, undertook a systematic study of the effect of oxygen exposure on AA to include measurement of tissue SOD levels in nonpulmonary target organs. These results, to be reported in detail elsewhere, indicated that hyperbaric oxygenation does indeed ameliorate adjuvant arthritis, but that the effect is probably not related to induction of SOD.

During the course of these studies, we noted that the serum of many of these animals had a greenish color following the animals' oxygen exposure, and this in turn led us to measure serum and plasma ceruloplasmin (CP), which we found to be consistently elevated. In this paper, we report the results of a systematic study of the effect of hyperoxia on rat serum CP, with concomitant measurement of lung antioxidant enzyme levels, control observations on hyperbaric air, and measurement of other serum factors indicating that the increase in CP levels is a relatively specific phenomenon and not merely a reflection of systemic inflammation.

Materials and Methods. *Oxygen exposure.* All experiments were performed with female Lewis rats initially weighing 80–100 g. Two exposure regimens were utilized. For hyperbaric oxygenation, which we refer to as HBO, a semiautomatic chamber was constructed which delivered the intermittent exposure regimen described in (2) which ameliorates AA, namely 6 hr of 100% oxygen at 2 atm, followed by 15 min of decompression at 1 psi/min, followed by 18 hr of normobaric room air, followed by gradual recompression, etc. The chamber housed 18 rats comfortably and operated unattended over weekends as required. Animals could be maintained in this manner for as long as 19 days without substantial mortality. In one control experiment, the chamber was used to deliver hyperbaric air by connecting the inlet tubing to an air tank rather than an oxygen tank.

Alternatively, in some experiments, rats were exposed to continuous (24 hr/day) oxygen at a concentration of 85% for periods up to 7 days. This regimen was referred to as an adaptation program (AD), since this

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type of exposure will produce "adapted" rats, i.e., rats capable of surviving subsequent exposure to otherwise lethal 100% oxygen (7). In both cases, control animals were maintained in adjacent facilities in normobaric room air, and all animals had continuous free access to normal rat chow and to water.

Sample preparation. Blood was obtained from the retro-orbital sinus under light anesthesia (sodium pentobarbital, 25 mg/kg ip); in a typical experiment, specimens were obtained at Days 4, 7, and 10 of O_2 exposure. The samples were allowed to clot at room temperature, centrifuged at 500*g* for 15 min, and then frozen for assay of ceruloplasmin (CP), total protein, copper, iron or serum SOD-like activity. In some experiments, citrated plasma was collected from the abdominal aorta and used for assay of plasma CP, fibrinogen, sulfhydryl groups, and total protein; similar samples were used for determination of the erythrocyte sedimentation rate in the standard clinical manner (modified Westergren).

For tissue collection, the rats were anesthesized with sodium pentobarbital 40 mg/kg ip and a midline incision was made through which the portal vein was catheterized; blood was also collected from the abdominal aorta at this time. Immediately after exsanguination, the aorta was severed and the lungs were perfused with phosphate-buffered saline at 37°C to remove red blood cells. The thoracic cavity was opened, the heart transected, and a catheter introduced into the right atrium through which additional flushing of the lung via the pulmonary artery was performed. The lungs, which were usually clean and white after this procedure, were removed, debrided, weighed, and homogenized with 9 vol of 0.05 *M* phosphate buffer containing 0.1% Triton X-100, pH 7.8 in a standard tissue homogenizer at full speed for 10 strokes. One aliquot of the homogenate was retained for DNA determination, and a second was centrifuged at 500*g* for 10 min in the cold to remove debris and then refrigerated overnight for assay of catalase (Cat), glutathione peroxidase (GPx), and total protein. The remaining unspun homogenates were frozen at −20°C for 7–10 days, thawed, and centrifuged at 20,000 rpm for 60 min at 4°C. The supernatants were dialyzed in the cold overnight against 0.05 *M* phosphate buffer, pH 7.8, and then used for assay of SOD activity.

Assay techniques. Serum and plasma CP levels were measured with the *ortho*-dianisidine dihydrochloride method in 0.1 *M* acetate buffer, pH 5.0, and results were expressed as international units based on the difference between absorbance readings at 5 and 15 min after incubation with substrate (9). Serum copper and serum iron were measured by atomic absorption spectroscopy. Total protein was assessed with the Lowry technique (10). Plasma fibrinogen was estimated by quantitative radial immunodiffusion using commercial antiserum to rat fibrinogen (Cappel Laboratories, Cochranville, Pa.); pooled normal rat plasma (from animals not involved in the experiment) was used as a standard in various dilutions, and the results were expressed for control or oxygen-exposed specimens in arbitrary units based on the value observed for the pooled normals. Plasma sulfhydryl groups were determined using DTNB (11).

Lung SOD was assayed by measuring the effect of the extracts on the superoxide-mediated reduction of cytochrome *c* produced by the action of xanthine oxidase (Sigma) on hypoxanthine. The SOD assay was performed at both pH 7.8 and pH 10.0 in the presence of 10^{-5} *M* KCN under the conditions described by McCord and Fridovich (12) as modified (13). Serum SOD-like activity was assessed by using neat serum in the same assay system. Catalase was measured with hydrogen peroxide as substrate (14), and GPx was determined by coupling the reaction of the enzyme and cumene hydroperoxide to glutathione reduction with NADPH oxidation by glutathione reductase (15). DNA was measured by the diphenylamine method (16).

Statistical analysis was performed with Student's *t* test and/or standard correlation coefficient as appropriate.

Results. Both the HBO and the AD oxygen exposure regimens led to increased levels of serum CP (Fig. 1). The differences between oxygen-exposed animals and room air controls were statistically significant at 7 or 8 days in over a dozen experiments, and in some cases, significant changes were noted as early as 4 days (data not shown). In every case, total serum protein was unchanged. There was a highly significant correlation

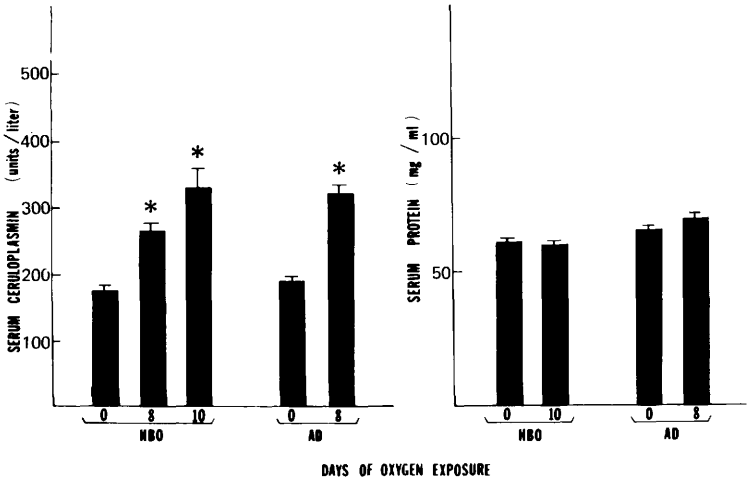

FIG. 1. Effect of oxygen exposure on serum ceruloplasmin levels in rats. Intermittent hyperbaric oxygen was administered for 8 or 10 days (HBO) or animals were maintained continuously for 8 days in 85% oxygen (AD). Total serum protein was unchanged (right panel). Bars indicate means ± SEM. $*P < 0.001$.

between serum CP and total serum copper (Fig. 2).

Both oxygen regimens produced increases of lung antioxidant enzymes, provided that the data were expressed as enzyme units per lung. Figure 3 shows representative data for the HBO regimen; similar findings were noted with AD. The differences were noted at 7 days, although statistical significance was not attained until 10 days in some cases. Total SOD always increased, and, in general, the rise in Cu–Zn enzyme averaged only 20–35% whereas the manganese enzyme levels more than doubled; this distinction has been dealt with in detail by others. Lung total protein also increased, and in confirmation of the findings of others, we too, noted that if the enzyme assay data were expressed as units per milligram of protein, statistical significance was often abolished. Comparison of Figs. 1 and 3 reveals a temporal relationship between the rise in serum ceruloplasmin and the rise in lung oxygen scavengers. A statistically significant correlation was apparent when serum CP was assessed as a function of lung SOD (Fig. 4).

Hyperbaric air treatment had no effect on lung SOD or serum CP. Measurements of plasma –SH groups, ESR, serum iron, and plasma fibrinogen revealed no difference between oxygen-exposed and control animals (Table I). When plasma CP was measured instead of serum, the hyperoxic enhancement effect was still noted and serum values were deemed to be interchangeable with plasma (data not shown). When the apparent SOD-like activity of serum was measured by adding serum to the standard xanthine oxidase/cytochrome *c* assay system, there was no statistically significant difference in activity between hyperoxic and control specimens, nor was there a correlation with serum CP levels.

Discussion. Ceruloplasmin is the greenish-blue, copper containing α_2-globulin of mam-

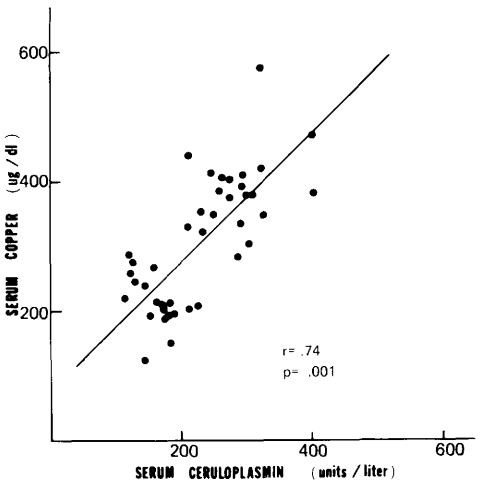

FIG. 2. Correlation of serum copper (determined by atomic absorption) with serum ceruloplasmin (oxidase activity) in blood samples from animals after intermittent hyperbaric oxygenation.

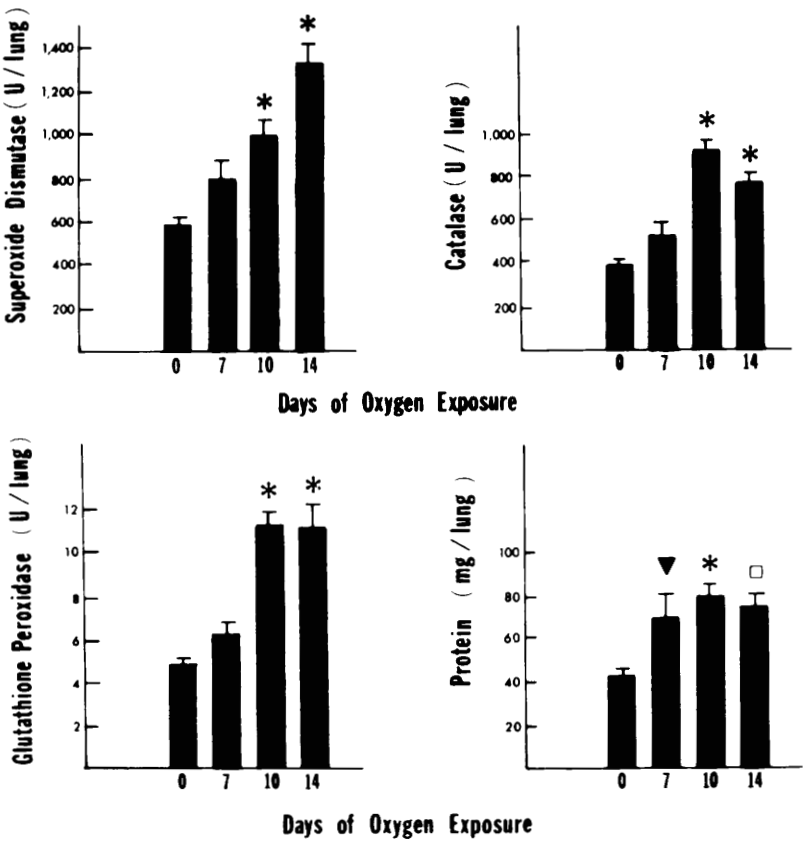

FIG. 3. Lung antioxidant enzymes following intermittent hyperbaric oxygen exposure for 0, 7, 10, or 14 days. Lung wet weights also increased in parallel. Bars indicate means ± SEM. $^{*}P < 0.001$; $^{\square}P < 0.01$; $^{\blacktriangledown}P < 0.05$.

malian plasma. It is a single polypeptide chain of molecular weight 134,000 containing (probably) six copper atoms per molecule (0.34%). Its properties have been the subject of two recent and comprehensive reviews (17, 18). CP possesses oxidase activity toward a variety of substrates, and this property is widely used in its assay. There is growing evidence that the physiological role of CP may relate to its action as a ferroxidase (vide infra). CP accounts for 95% of the copper content of normal plasma. Since serum CP has been reported to be 15–40% lower than plasma CP in cows and sheep (19), we measured the latter in one experiment and confirmed the finding of hyperoxic increases of either serum or plasma values.

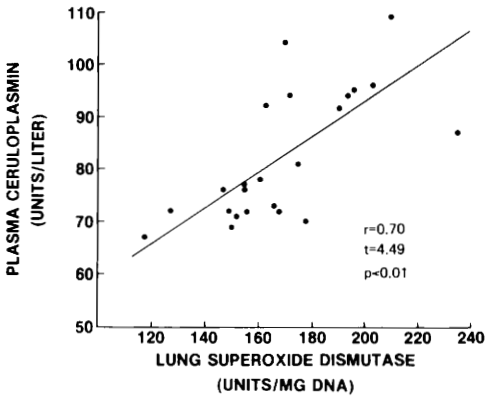

FIG. 4. Correlation of plasma ceruloplasmin and lung SOD activity after hyperbaric oxygen exposure.

CP is present in most mammalian sera, is low in neonates, and is markedly elevated in pregnancy or following ingestion of oral contraceptives. The most widely acknowledged factor affecting serum CP levels is the protein's behavior as an acute phase reactant,

TABLE I. MISCELLANEOUS RAT BLOOD MEASUREMENTS AFTER EXPOSURE TO INTERMITTENT HYPERBARIC OXYGEN FOR SPECIFIED INTERVALS

	Days of exposure			
	0 (Air controls)	3	7	10
Plasma ceruloplasmin				
(units/liter)	71.9	73.4	90.0*	93.4*
(units/g protein)	1.62	2.00*	2.20*	2.07*
(% control)	100.0	123.4*	135.8*	127.8*
Total plasma protein (mg/ml)	44.4	36.6	40.9	45.0
Plasma -SH groups (m*M*)	0.28	0.29	0.35	0.31
Plasma fibrinogen (units/ml)	190.0	216.0	217.0	232.0
Serum iron (μg/dl)	115.8	n.d.	106.9	n.d.
Sedimentation rate (mm/hr)	0.60	1.40	0.50	0.40

Note. Means are of at least eight determinations. n.d., Not determined.
* $P < 0.05$, all other differences were nonsignificant.

with elevated levels occurring in conjunction with many stimuli, including infection, endotoxin injection, neoplasia, liver disease, and inflammatory disorders such as rheumatoid arthritis. Of great interest is the observation that when CP behaved as an acute phase reactant, it correlated with fibrinogen (20); in the present study, the CP inductive effect was specific for the copper protein alone and other phase reactants were not elevated.

In the current study, the close correlation between elevated CP levels and serum copper confirmed the results obtained by oxidase assay. However, we did not measure ceruloplasmin immunochemically, and we therefore cannot state that there was an absolute rise in total ceruloplasmin protein distinguishable from either enhanced specific activity or greater affinity for copper. There was no change in plasma fibrinogen, total serum protein, serum -SH groups, or erythrocyte sedimentation rate, suggesting further a specific rather than nonspecific inductive effect. Histologic changes in the lungs of HBO animals are quite mild, the animals do not manifest systemic signs of oxygen toxicity, and they even gain weight. Since they are not systemically ill, there is little reason to suspect a generalized inflammatory stimulus to CP induction. Rats are indeed susceptible to such an effect; for example, carrageenan foot edema or pleurisy induces rat CP (21).

Whole animal exposure to sublethal concentrations of oxygen leads to enhanced levels of all three pulmonary antioxidant enzymes; this is well known in the rat (3–7) and has recently been noted in the piglet as well (22). In most such studies, the results are consistent only when the data are expressed as units of enzyme activity per whole lung; and when the data are normalized for protein, DNA, cell number, etc., the relationship often loses statistical significance. Isolated cells can also be induced to form more SOD, and this has been demonstrated in both alveolar macrophages (23) as well as neutrophils (24). When Type II pneumocytes were cultured from oxygen-exposed animals, a similar enhancement was noted (25). Of interest is the fact that endotoxin administration, which both protects against oxygen toxicity and induces antioxidant enzymes, also leads to enhanced serum CP levels (26, 27). Pulmonary endothelial cells are susceptible to damage by oxygen radicals (28), but if the animals are first adapted, they develop resistance (29); isolated endothelial cells can also be induced to develop SOD under hyperoxic conditions (30). Thus, it appears that the organism responds to the threat of oxygen toxicity by enhancing its levels of radical-scavenging enzymes in susceptible target tissues. The correlation demonstrated in Fig. 4 suggests that two separate organ systems, both the lung and the liver (where ceruloplasmin is presumably manufactured), sense the same stimulus of threatened oxygen toxicity and respond in parallel fashion by production of antioxidant defenses.

Ceruloplasmin is another potential antioxidant substance whose levels are enhanced after oxygen exposure. Ceruloplasmin has long been recognized as one of the plasma antioxidant substances, but only recently has it been noted that this protein also can scavenge superoxide and related oxygen radical species. CP inhibited the superoxide-mediated reduction of cytochrome *c* and nitroblue tetrazolium (31, 32); this occurred by stoichiometric reaction rather than catalytic activity (contrast SOD). ESR studies have been reported to show dismutation of the superoxide radical by CP (33), but its action as a true dismutase has been questioned (34). CP protects substrates such as erythrocytes (35) or liposomes (36) from oxy radical damage.

Although CP is not as powerful a scavenger of oxy radicals as is SOD, the latter is almost totally an intracellular enzyme, whereas CP is primarily found extracellularly where it might prove to be one of the major defenses against oxy radical tissue damage. Marklund (37) has described an extracellular SOD-like enzyme which curiously has the exact same molecular weight as CP, namely 134,000, although it appears to have much greater activity and to be a true dismutase. Injection of CP into rats has been reported to ameliorate inflammation induced by injection of urate crystals (38). CP is well known to be increased in serum from patients with rheumatoid arthritis (39). The mechanism by which hyperoxia suppresses adjuvant arthritis in rats is unknown, but our preliminary data indicate that induction of tissue SOD is not a major factor. Ceruloplasmin may be a component of a complex antioxidant defense system which functions extracellularly to inhibit inflammation and ameliorate oxygen radical induced tissue damage. One mechanism by which this might occur relates to ceruloplasmin's function as a ferroxidase. By keeping free iron in the ferric state, the potential reactivity of superoxide-generated ferrous ion with hydrogen peroxide via a Fenton mechanism, leading to hydroxyl radical generation, could be lessened (40).

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