Effects of CO_2 exposure on distribution of various forms of iron and copper in guinea-pig tissues[1]

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Summary. The effects on iron and copper distribution and metabolism of exposure to high levels of CO_2 were studied in the guinea-pig. Mature, male animals were placed in an atmosphere of 15% CO_2, 21% O_2 (balance N_2), and sacrificed from 1 h to 1 week thereafter. Total iron and copper concentrations of blood, liver, spleen and bone, as well as concentrations of heme and ferritin iron, were measured together with blood hematocrit, reticulocytes, plasma hemoglobin, plasma ceruloplasmin and copper concentrations. The results show clearly that rapid and sustained red cell damage or hemolysis ensued several h from the start of CO_2 treatment. This resulted in loss of iron and copper from the blood, an influx of both elements into liver, spleen and bone, and a rise in plasma ceruloplasmin. Influx of iron into liver and spleen caused an accumulation of ferritin, the main site for iron storage in cells. Following the effect on red cells, there was an accumulation of heme iron, and a decreased hematocrit, best explained by a depressed activity of the reticuloendothelial and erythropoietic systems. A period of adaptation succeeded these events, in which all blood parameters and most tissue values returned to normal, despite the continuing presence of high CO_2. The only changes not reversed were the elevations in liver, spleen and bone iron stores. These remained high, with a net accumulation of > 2 mg iron, or 3–4 times more than originally present. The results indicate that at least in the guinea-pig, high CO_2 exposure results in red cell damage and other events leading to an accumulation of additional iron in the body; also, that iron accumulated as ferritin and hemosiderin in liver and spleen may not be readily available to restore blood hemoglobin concentrations on an acute basis.

Introduction

In mammals, the majority of iron is found in hemoglobin within the red cells of the blood. Other cells contain iron mainly stored in ferritin and hemosiderin, with smaller amounts in iron-containing enzymes most of which are hemoproteins. [In a homogenate made of whole tissue, a small portion of the iron will also be from residual blood in the tissue].

Ferritin is a large, multisubunit protein considered with hemosiderin the main site for iron storage in cells. Its adaptive response to the influx of iron has been well studied and involves the induction by iron of an increased rate of ferritin synthesis, involving activation of specific mRNA[21,35]. Hemosiderin is defined biochemically as insoluble, non-ferritin, non-heme iron, and there is some evidence it may be a degradation product of ferritin[34]. In spleen and liver, the proportion of hemosiderin to ferritin iron will vary with iron status and vitamin C, being increased in iron overload[34] and ascorbate deficiency[28]. In the case of the scorbutic guinea-pig, the larger proportion of hemosiderin is associated with a decreased release/return-flow of iron from the spleen to the plasma.

About 1% of the iron in blood hemoglobin is released and replenished daily, in conjunction with red cell turnover. Old or damaged red cells are removed by reticuloendothelial (RE) cells especially of the spleen and liver[22]. After internal digestion, the iron is released back to plasma transferrin, which returns it to the marrow for reincorporation[11]. Some of the iron from degraded hemoglobin is stored in ferritin and hemosiderin in RE cells[4,5]; some is secreted into the plasma as serum ferritin[32], which finds its way mainly to liver parenchymal cells[33]. If hemolysis of red cells occurs, free hemoglobin and heme iron appear in the plasma, and are carried directly to liver hepatocytes[11,33], on haptoglobin and hemopexin, respectively. This results in increased iron and ferritin concentrations in the liver. Damaged whole red cells are taken up separately, as already noted, increasing the accumulation of iron in RE cells of spleen and liver. Thus, hemolysis or red cell damage results in the accumulation of extra iron in spleen, liver and other organs of the RE system, and much of this iron is stored at least temporarily in ferritin and hemosiderin of spleen and liver. Increased loss of red blood cells from the blood also stimulates the erythropoietic process in marrow, and will enhance dietary iron absorption[18].

Ferrokinetics and red cell metabolism require the participation of copper[20, 24]. The exact nature of this involvement is unclear but may depend upon actions of ceruloplasmin, the principal copper-containing protein of plasma, to enhance the flow of iron from liver storage sites to the marrow[23,25]; also, on the need for copper incorporation into cuproproteins in red cells[17].

In extensive studies on the effects of long-term hypercapnia in guinea-pigs[3,29–31], a variety of physiological and biochemical changes were observed which

prompted the question whether alterations in ferrokinetics and red cell metabolism were occurring. Upon exposure to 15% CO_2, 21% O_2 (balance N_2), guinea-pigs exhibited a transient, uncompensated, respiratory acidosis, during which blood pH fell to 7.0; subsequently, an adaptation to respiratory acidosis, in which the pH rose to 7.25[29]. Red cell pH also dropped markedly[31], and there were temporary shifts of blood out of liver, brain, muscle and skin[30]. We have now examined the total iron, heme and ferritin iron contents of liver, spleen and bone at different times after exposure of adult guinea pigs to 15% CO_2 (normal oxygen); also, the concentrations of copper in blood, liver and bone. The results provide direct evidence of enhanced red cell breakdown and an increase in the body iron burden.

Methods

Animals and treatment. Adult, male Hartley guinea-pigs, weighing 400–500 g (Charles River Labs, Wilmington, MA) were exposed to, and maintained in, an atmosphere of 15% CO_2 and 21% O_2, the balance N_2, as previously described[31]. Animals were killed 1 h to 7 days after the start of exposure, and in some instances after 1 day of recovery in normal air after 7 days of exposure to 15% CO_2. Whole blood, liver, and spleen, as well as femurs, were removed and frozen. In some cases, blood was separated into plasma and cells before freezing, and hematocrits measured. Frozen samples were later thawed, the tissue homogenized in 4 vol. distilled deionized water, and analyzed for iron and copper content, as described below.

Prior to sacrifice, the animals received 40 mg pentobarbital/kg b.wt s.c. and were returned to the CO_2 exposure chamber within 4–5 sec. The anesthesia was usually effective after approximately 5 min, at which time the animals were taken out of the exposure chamber and immediately placed under a mask through which they breathed the same CO_2 gas mixture to which they had been exposed. Blood samples were drawn from the abdominal aorta, and hematocrits were determined by the microcapillary method. Reticulocytes were counted after cresyl blue staining[7]; hemoglobin in plasma was assayed by the benzidine technique[6].

Assays of iron and ferritin content. Aliquots of whole blood, plasma, and tissue homogenates were assayed for total iron content by procedures previously described[33]. In the case of femurs, the clean whole bone was dissolved in 5 ml concentrated HNO_3 + 1.5 ml $HClO_4$ prior to a double wet ashing of the mixture and the usual iron determination[33]. For liver and spleen, heme iron contents were determined on aliquots of homogenate as previously described[33]. Portions of homogenate were heated to 70 °C for 10 min, and supernatants analyzed for ferritin iron, using the standard immunological procedures applied in this laboratory[16]. Rabbit anti-serum directed against horse spleen ferritin was found to cross-react and precipitate guinea-pig ferritins with about the same efficiency as rat ferritins. This was checked by electrophoresis[19].

Cooper and ceruloplasmin determinations. The total copper content of plasma, liver, and bone (femur) was determined using aliquots of the same digests used to analyze total iron (see above). Reagent blanks were always included and subtracted from the appropriate absorption values. Liver copper concentrations were determined by the method of Giorgio et al.[9], while the much lower levels in plasma and bone were assayed by anodic stripping voltammetry, as previously described[15]. Ceruloplasmin oxidase activity was assayed by the classic procedure of Houchin[12] and Ravin[26], using p-phenylenediamine as substrate, and 0.010 M EDTA to prevent non-specific substrate oxidation[13]. Activity was calculated using the molar extinction coefficient of Rice[27] (1.91×10^6/cm at 540 nm) for the purple product of the reaction, all as previously reported[15]. Values are given in terms of 10^5 IU/ml plasma.

Results

Table 1 shows the effects of prolonged exposure to 15% CO_2 (21% oxygen) on plasma hemoglobin content, blood hematocrit and the number of circulating reticulocytes. Hemoglobin was detected in significant amounts in plasma after 1 h, indicating the occurrence of some red cell damage or hemolysis by this time. The number of circulating reticulocytes rose significantly by 6 h and remained elevated for at least the next 2.5 days, before returning to control levels at 7 days. Blood hematocrit showed a significant (10%) drop by 24 h of exposure, remained low for the next 2 days, and returned to normal by 7 days.

Figure 1 presents the concentration of iron in whole blood, spleen, liver and bone. Since there were no significant changes in organ weights, this reflects the total iron content of the organs. In parallel with the fall in hematocrit over 1 day of CO_2 exposure, there

Table 1. Effects of prolonged exposure to 15% CO_2 in 21% O_2 (balance N_2) on plasma hemoglobin, blood hematocrit and reticulocyte counts. All values are means ± SEM, with the number of determinations (animals) in parentheses.

Time after CO_2 exposure	Plasma hemoglobin (μg/ml)	Blood reticulocytes (1000/mm³)	Blood hematocrit (%)
0 h	0.00 (10)	17.1 ± 1.4 (10)	44.3 ± 0.8 (10)
1 h	0.45 ± 0.25 (6)	22.2 ± 2.7 (5)	43.6 ± 0.8 (6)
6 h	0.00 (6)	54.2 ± 2.7 (5)*	45.1 ± 0.8 (6)
1 day	0.00 (6)	34.3 ± 3.2 (6)*	39.7 ± 1.9 (6)**
3 days	0.00 (5)	49.2 ± 5.8 (6)*	40.3 ± 2.2 (5)
7 days	0.00 (5)	24.0 ± 5.6 (6)	43.1 ± 0.9 (5)

* $p < 0.01$ for difference from 0-time; ** $p < 0.05$ for difference from 0-time.

was a significant (20%) decrease in total blood iron content. In contrast, concentrations of iron in spleen, liver, and bone rose markedly already by 6 h.

Spleen was the organ most affected. It showed a 4-fold rise in total iron concentration, while the liver and bone only doubled their iron contents. On a whole organ basis the liver was the recipient of about 1100 µg iron; spleen received about 300 µg in the same period. Extrapolating from the results for the femur (which represents 8% of the skeleton and marrow), bone accumulated another 2300 µg (150 µg/g × 12.5 × femur weight).

During the later phases of CO_2 exposure (from 3 to 7 days) there was a fall in the iron content of the spleen (fig. 1), indicative of a trend of return to pre-exposure values. There was no such trend in the liver which continued to maintain abnormally high concentrations of iron. The assays on bone were fewer and more variable, but also suggested no ultimate return to pre-exposure values. [The drop at 3 days may have reflected an increase in the rate of hematopoiesis, the final rise a reflection of increased body iron (see below).]

The influx of iron into the liver by 6–8 h was paralleled by a 3-fold increase in ferritin iron concentrations (fig. 2) which remained over the entire period of study. Whereas at zero time, half of the total non-heme iron (total minus heme iron) was accounted for by ferritin, this increased to two-thirds after exposure to CO_2. Exposure to normal air for 1 day did not reverse the situation. In spleen, an accumulation of

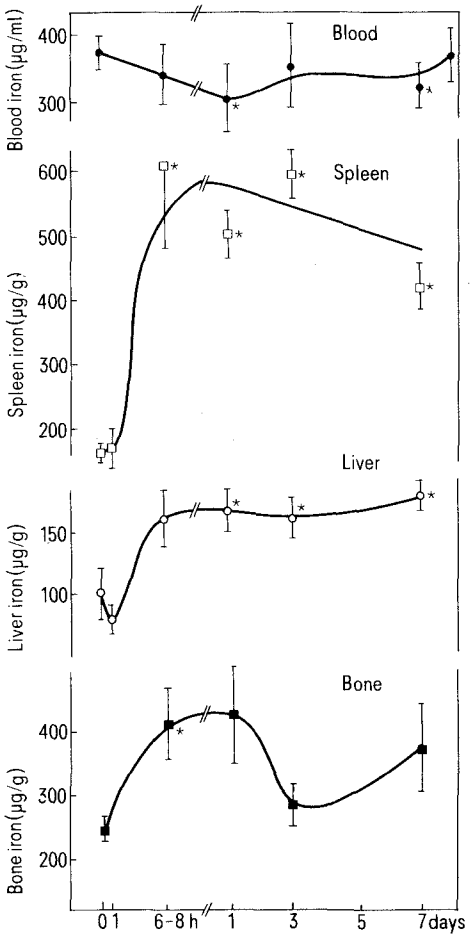

Figure 1. Concentrations of total iron in blood, spleen, liver and bone at various times after the start of exposure of guinea-pigs to 15% CO_2. The points shown represent mean values (µg Fe/g or ml) for 3 sets of experiments (9–17 animals per point), except in the case of bone where only 1 set was available (3–6 animals). Bars represent ±SEM. Values significantly different from 0-time values ($p < 0.05$ or less) are starred (*). Liver and spleen weights did not vary and averaged 21.8±3.6 (52) and 0.77±0.18 (52) g, respectively (mean ±SD; No.).

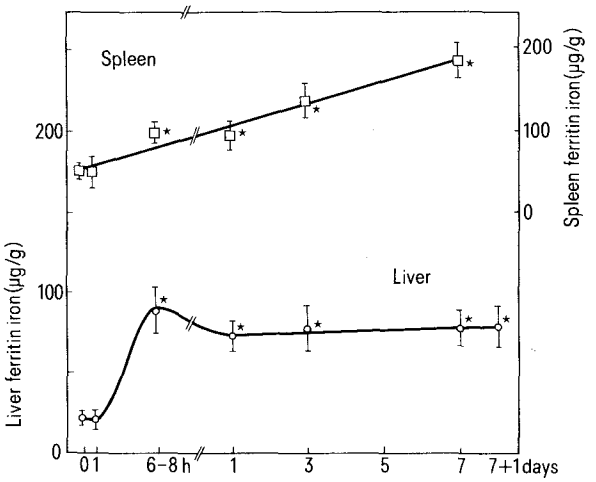

Figure 2. Concentrations of ferritin iron (µg/g) in spleen and liver at various times after the start of exposure of guinea-pigs to 15% CO_2. Details as in figure 1.

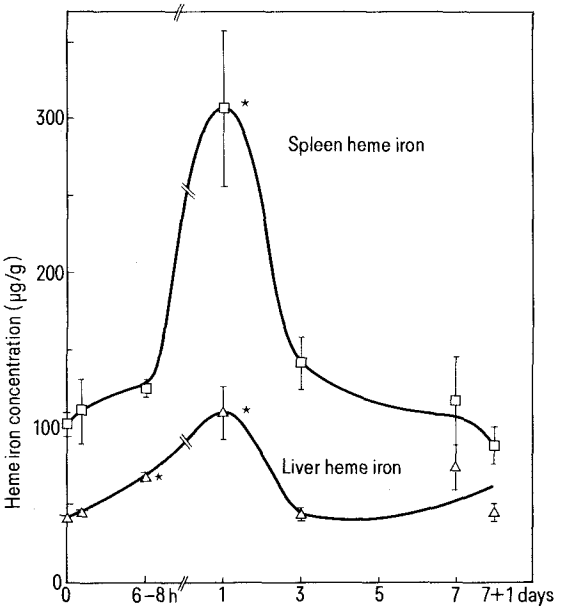

Figure 3. Concentrations of heme iron (µg Fe/g) in spleen and liver as function of time guinea-pigs were exposed to 15% CO_2. Mean values ±SD are fro 2 sets of experiments (6–14 animals per time point). Other details as in fig. 1.

ferritin also occurred following the increased influx of iron, but this change was much more gradual. It progressed during the period of CO_2 exposure, reaching a level 3.5-fold above the normal by 7 days. In the process, the percentage of total iron in ferritin went from about 30%, at 0-time, to about 20% by 6–8 h, and back to 44% by 7 days.

Following the accumulation of iron in liver and spleen, there was a delayed, transient accumulation of heme iron in these tissues (fig. 3). In liver the concentration doubled, and a much larger increase occurred in the spleen. In both cases, values returned to normal by 3 days after the start of CO_2 treatment.

Copper concentrations of plasma liver and bone, as well as ceruloplasmin were also examined. Liver and bone (fig. 4) accumulated copper in the early phases of exposure. In the liver the maximum increase occurred by 6 h, in parallel with the accumulation of iron in that organ. In bone, copper concentrations increased to the same extent as in liver during the first 6 h, but then continued to rise, reaching a level 2.5-fold above the normal by 24 h (fig. 4). This maximum was after the maximum increase in iron concentrations in that organ (fig. 1) but at the time of maximum heme accumulation in spleen and liver (fig. 3). Increases in tissue copper concentrations were transient (as for heme) and in liver appeared to follow inversely the changes in plasma copper concentrations (fig. 4). Quantitatively, the loss of plasma copper did not entirely account for the increase in liver copper observed over the same period (assuming blood volumes of 43 ml and a liver weight of 12 g).

Ceruloplasmin concentrations (fig. 4), as measured by p-phenylenediamine oxidase activities, showed an initial 15% drop (by 6–8 h) followed by a substantial rise, by 1 day after the start of CO_2 treatment. This elevation persisted for a few days and was followed by return to normal levels. The magnitude of the initial fall in ceruloplasmin was not as great as that for plasma copper concentrations (fig. 4). If as in rat and man, ceruloplasmin accounts for two-thirds of plasma copper[14], this indicates that the non-ceruloplasmin portion of plasma was preferentially depleted in the early period of CO_2 exposure.

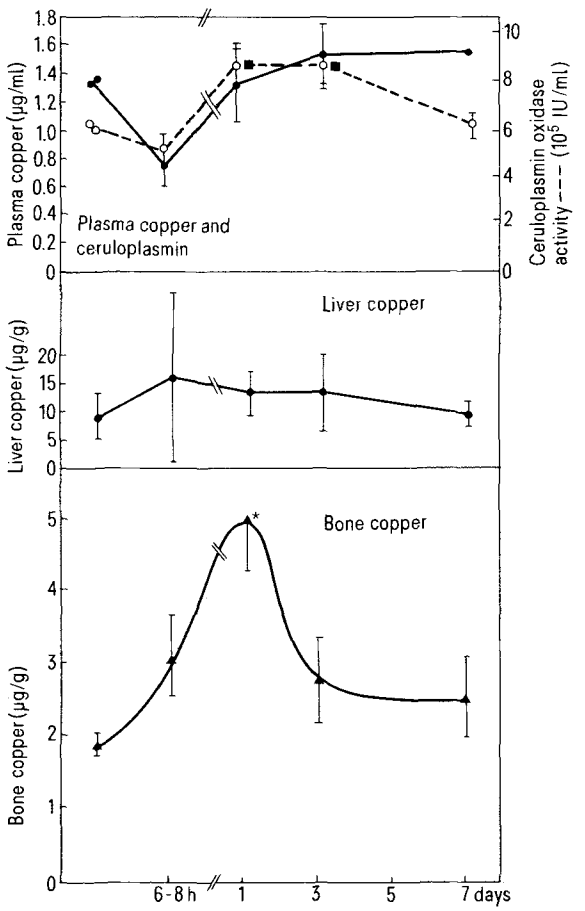

Figure 4. Concentrations of copper (µg/g) (—) in plasma, liver and bone of guinea-pigs exposed to 15% CO_2. Details as in figure 1, for 1 set of 2–6 animals (determinations). The top figure also shows values for plasma ceruloplasmin oxidase activity (----), Mean ±SEM, in 10^{-5} IU/ml (p-values relative to 7-day mean).

Discussion

The results provide evidence for large scale changes in the distribution of iron and capper in tissues upon acute exposure of guinea-pigs to high concentrations of CO_2 in the presence of normal O_2. These effects are of potential interest to our understanding of the effects of underwater diving and the submarine environment on metabolism[29], and the long-range effects of such treatment on nutrient status and health. They provide direct evidence for the occurrence of red cell damage or hemolysis, and for the promotion by high CO_2 of the accumulation of larger body iron stores. More indirectly they suggest a temporary suppression of reticuloendothelial function, followed by adaptation and a return of blood parameters to normal values. The sequence of events observed and the probable changes underlying them are summarized in table 2.

The sequence of events observed fits well into our picture of ferrokinetic responses to erythrocyte damage or hemolysis: the immediate appearance of hemoglobin in plasma and a previously determined increase in red cell osmotic fragility[31], perhaps induced by the fall in blood and red cell pH. Also, the deposition by 6–8 h of large amounts of iron (and copper) in liver, spleen and bone; the increased synthesis and accumulation of ferritin – responsive to influx of iron into parenchymal cells; the increase in reticulocytes. Similarly, the fall in hematocrit and total blood iron by 24 h.

The accumulation of iron in liver, spleen and bone was not simply the reflection of an increased blood content of these organs. Previous studies had shown

Table 2. Summary of events recorded and underlying the changes observed. Starred (*) events are those for which data are provided. Others are based on previous knowledge (see text)

0-time	Exposure to high CO_2
	↓
by 1 h	↑Red cell fragility → Hb leakage or cell lysis
	Hb appears in plasma*
by 6–8 h	↑Uptake of damaged red cells by RE system
	↑Fe/Cu in liver, spleen, bone*
	↑erythropoietin release
	↑reticulocyte release*
	(↑dietary Fe uptake → marrow and new red cells)
	(↑erythropoiesis)
	↓plasma Cu*
by 24 h	Continued uptake of damaged red cells
	↓hematocrit/total blood Fe*
	↑plasma volume*
	↑ceruloplasmin in plasma*
by 3 days	RE cell function has caught up with need
	↓heme/Hb in liver/spleen*
	Continued enhanced erythropoiesis
	reticulocytes still high*
	hematocrit still low*

had been exceeded, at least temporarily. As there was no additional accumulation of total tissue iron, there appeared to be no change in the rate of iron release from the RE cells of these organs, a release balanced by the influx of damaged and old erythrocytes.

The depression of hematocrit, and the elevation of iron and copper in bone would also be consistent with a reduced erythropoietic activity of the bone marrow, in accord with some earlier studies in mice where the erythropoietic response to a hypoxic environment was blunted by an atmosphere with 5% CO_2[8]. However, the enhanced loss of erythrocytes induced by the hypercapnia would be expected to enhance the release of erythropoietin, in order to induce a compensatory increase in erythropoiesis. The elevation of blood reticulocytes which persisted through day 3 implies that enhanced erythropoiesis is indeed occurring, but also that it was insufficient to restore normal levels of red cells by this time. The persistence of higher than normal levels of iron and copper in bone at 24 h is consistent with an increased need for

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Mini-Review

Molecular mechanisms in the action of imipramine

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For many years our understanding of how imipramine relieves some symptoms of depression and our theories on the etiology of affective disorders have progressed 'pari passu', almost as if pharmacology was leading the way. This association began when it was discovered that imipramine blocks the uptake of catecholamines and relieves the symptoms of depression. This observation suggested that imipramine