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Pulmonary Antioxidant Enzyme Maturation in the Fetal and Neonatal Rat. II. The Influence of Maternal Iron Supplements upon Fetal Lung Catalase Activity

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Summary

It was observed that the lung catalase activity of premature (day 21 of gestation; term = 22 days) rat pups is affected by maternal iron intake. Pups from control dams receiving Purina Lab Chow and water *ad libitum* have only 50% of the lung catalase activity of pups from dams who received 1 mg/kg parenteral iron dextran daily from day 7 to day 20 of gestation. Other oxygen-protective enzymes, copper-zinc and manganese superoxide dismutase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase, were unaffected by maternal iron supplements.

Abbreviations

CuZn SOD, copper zinc superoxide dismutase
CAT, catalase
G-6-PD, glucose-6-phosphate dehydrogenase
GPx, glutathione peroxidase
Mn SOD, manganese superoxide dismutase
HBSS, Hanks' balanced salt solution

Undernutrition is reported to enhance pulmonary oxygen toxicity (10). One potential contributing factor is a loss of metalloprotein antioxidant enzymes activities upon metal depletion. This has been reported with copper (4) for CuZn SOD, with manganese (6) for Mn SOD, with iron and selenium (18) for GPx, and with iron (20) for CAT.

We have previously reported that the specific activity of lung catalase of gravid female rats fed Purina Lab Chow *ad libitum* declined with gestation (13). The fetuses of these animals demonstrated gestation-dependent increases in specific activity of CuZn SOD, Mn SOD, GPx, and G-6-PD in lung between day 18 and day 22 (term) of gestation (24). The specific activity of fetal lung CAT, however, increased between days 18 and 20 of gestation, but showed no significant increase between day 20 and term (24). Since the fall in maternal lung CAT activity towards the end of gestation was reversed by parenteral iron supplements, it is possible that the blunted increase in fetal lung catalase reflected limited iron transport across the placenta.

In this report, we examine the effect of maternal iron supplementation on fetal lung growth, and on the specific activity of oxygen-protective enzymes of fetal rats sacrificed prematurely on day 21 of gestation. Reported values have been corrected for enzyme activity contributed by contaminating blood by a modification of the method described by Cross *et al.* (5), using homologous blood supplements.

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MATERIALS AND METHODS

Female Sprague-Dawley white rats of 275–300 g (Charles River Inc., St. Constant, Quebec) were mated with male hooded rats and delivered to our animal quarters the following day. From day 7 to day 20 of gestation, the gravid rats were injected intramuscularly with either 1 mg/kg/day of iron dextran in isotonic saline or isotonic saline alone, or received no injections. On day 21 of gestation (term = 22 days), the animals were sacrificed by excess chloroform anesthesia; the fetuses were decapitated *in utero* to prevent respiration and immediately removed from the uterus. Each fetus was weighed and the carcasses were placed in HBSS at 4°C. The chest cage was opened from diaphragm to neck; then the heart and lungs were removed *en bloc* and kept in HBSS at 4°C. The heart and major airways were dissected off and the lungs from each fetus were washed with HBSS, blotted dry, and weighed. The lungs from each litter were then pooled, minced, and homogenized in 5 ml 50 mM potassium phosphate, 0.1 mM EDTA, pH 7.8 for 30 s at 4°C with a Polytron (Brinkmann Instruments, Inc., Westbury, NY). The homogenates were centrifuged at $1000 \times g \times 10$ min to remove fibrous material and the supernatants were stored at −70°C prior to analysis. Preliminary observations showed no loss of enzyme activity with centrifugation or a single freeze-thaw of homogenates.

Protein content was estimated by the Lowry method (19), and DNA was estimated fluorimetrically (7). CAT (EC 1.11.1.6.) was measured as described by Bergmeyer (2), GPx (EC 1.11.1.9.) as described by Beutler (3), and G-6-PD (EC 1.1.1.49.), CuZn SOD, and Mn SOD (EC 1.15.1.1.) as described by Hayatdavoudi *et al.* (14). A number of homogenates from each group also had CAT estimations performed in the presence of 10^{-9} to 10^{-6} M iron as ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$).

A separate group of day 21 fetuses were sacrificed and decapitated, and blood was collected from the neck vessels by gravity drainage. Aliquots of this blood were diluted 1:1000 with 0.1 M Tris-HCl, 1% Triton X-100, pH 7.0, and sonicated for 15 s at 4°C. The absorbance of 1 ml of fetal blood lysate was recorded from 350 to 650 nm before and after the addition of 100 μl of 0.1 M NaOH, 0.1 M $Na_2S_2O_4$, as described by Cross *et al.* (5) in a Gilford 252 spectrophotometer (Gilford Instruments, Oberlin, OH) with a Beckman DU optical system (Beckman Instruments, Fullerton, CA).

Aliquots (5, 10, 20, 30, or 40 μl) of the 1:1000 blood sonicate were added to 100 μl lung homogenate for each litter and made up to 1000-μl final volume with 0.1 M Tris-HCl, 1% Triton X-

100, pH 7.0, sonicated for 15 s on ice, and then centrifuged at 11,000 × *g* for 10 min. The "dithionite index" for each sample was calculated as described by Cross *et al.* (5), except that the wavelength used was 405 nm, by the formula: dithionite index $= A_{405} - (1.1 \times DTA_{405})$ where DTA_{405} represents the absorbance in the presence of 100 μl 0.1 M NaOH, 0.1 M $Na_2S_2O_4$ and the factor of 1.1 corrects for dilution. The dithionite index obtained by addition of blood sonicate was plotted for each sample and found to be indicative of successively increasing blood contamination. From this, the original blood contamination in the sample could be calculated by extrapolation from blood-contaminated dithionite index plots.

Aliquots of blood were also examined for CAT, CuZn SOD, Mn SOD, G-6-PD, and GPx activity and, from the previous calculations of blood contamination, it was possible to correct each lung enzyme specific activity for enzyme activity due to blood contamination.

All results are presented as means ± SEM unless otherwise stated, and statistical comparisons were made by analysis of variance. The basic parameters, and enzyme activities, of the saline-injected and noninjected control groups showed no significant differences ($p > 0.05$) and have been combined as a single control group.

Table 1. *Basic parameters of study groups*

	Control	+Iron
No. litters	9	7
Pups/litters	13 ± 1	12 ± 1
Body weight/pup (g)	5.2 ± 0.1	5.3 ± 0.2
Lung weight/pup (mg)	133 ± 3	134 ± 8
Body weight/lung weight ratio	39.1 ± 0.7	39.7 ± 2.2
Lung protein (mg/pup)	4.9 ± 0.2	4.9 ± 0.3
Lung DNA (μg/pup)	115 ± 16	103 ± 15
Protein/DNA ratio	48 ± 6	51 ± 6
Blood contamination (μl/g)	7.1 ± 1.8	9.5 ± 1.9

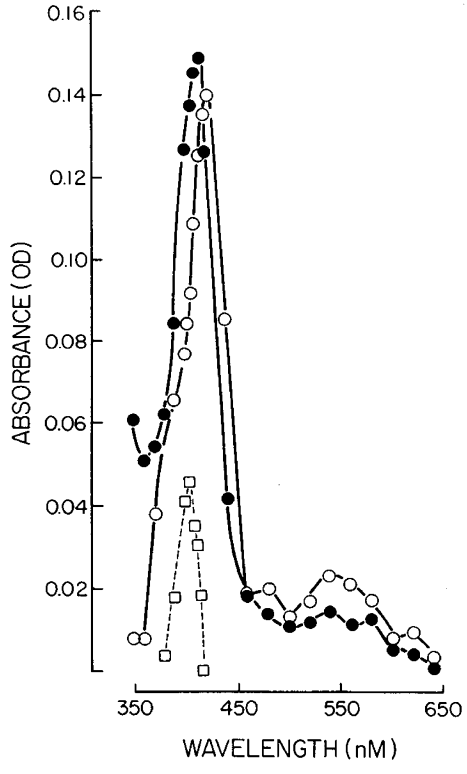

Fig. 1. The absorption spectra of 1:1000 day 21 rat fetal blood sonicate before (●) and after (○) the addition of dithionite. The peak at 405 nm (□) is the maximum of the difference of the two spectra and forms the basis of the dithionite-index.

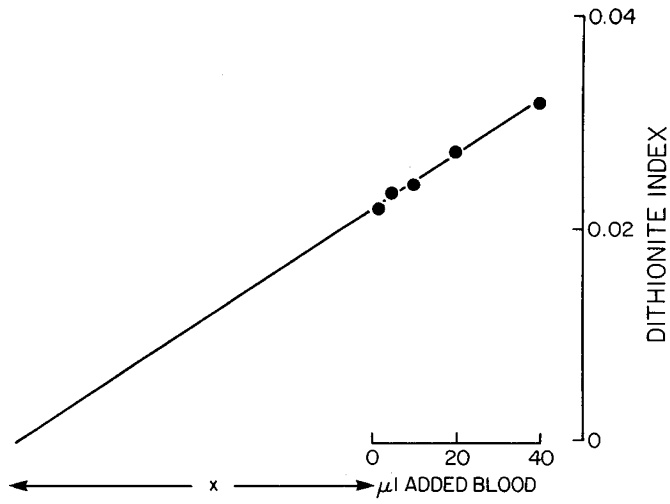

Fig. 2. Measurement of lung homogenate blood contamination. The *line* connecting the dithionite indices after the addition of 5, 10, 20, 30, or 40 μl of 1:1000 whole blood sonicate to fetal lung homogenate has been extrapolated to the baseline. The distance from this *intercept* to the zero point of the baseline (x) represents the absolute value of the contamination of the diluted lung homogenate with blood.

Table 2. *Per cent contribution of blood to lung homogenate antioxidant enzyme activity**

Group	*n*	CuZn SOD	Mn SOD	Catalase	GPx	G-6-PD
Control	9	4.6 ± 1.1	<1	4.5 ± 1.4	3.3 ± 0.7	20.1 ± 4.9
+Iron	7	4.7 ± 1.1	<1	3.9 ± 1.0	3.6 ± 0.7	22.0 ± 9.3
Combined groups	16	4.7 ± 0.8 (4.3)	<1	4.2 ± 0.9 (31.0)	3.4 ± 0.5 (3.6)	20.9 ± 6.3 (6.1)

* Values in parentheses are calculated from Cross *et al.* (5) for perfused adult lung homogenates.

RESULTS

The values for fetal lung weight, body weight, lung protein, lung DNA, and blood contamination are shown in Table 1. Values for fetuses of iron-injected mothers are not significantly different ($p > 0.05$) from those of controls.

The absorbance of fetal blood lysate from 350 to 650 nm is shown in Figure 1 before and after dithionite reduction (corrected for dilution). The absorption maximum which occurs at 405 nm in the spectral difference forms the basis of the dithionite index. In Figure 2, the effect of addition of 5, 10, 20, 30, or 40 μl of 1:1000 blood lysate on the dithionite index of aliquots of a lung homogenate is shown. Extrapolation of the plot line to zero generates a value of x, from which blood contamination can be directly calculated. Contamination was not significantly different for the control or treated groups.

Values for the contribution of blood contamination to enzyme activities are shown in Table 2 and compared with values for average perfused adult rat lung homogenates, reported by Cross *et al.* (5). Values for lung enzyme activities in each of the groups, after correction for contamination with blood, are shown in Table 3 where values are expressed as U/pup, U/g lung wet weight, U/mg protein, and U/mg DNA. Only lung CAT activity was significantly different between iron-supplemented and control groups ($V_1 = 1$, $V_2 = 14$: U/pup $F = 5.502$, $p = 0.034$; U/g

Table 3. *Influence of maternal iron supplementation on fetal lung antioxidant enzyme activity**

Enzyme	Group	*n*	U/pup	U/g wet weight	U/mg protein	U/mg DNA
CuZn SOD	Control	9	0.70 ± 0.07	5.33 ± 0.56	0.14 ± 0.02	6.92 ± 1.26
	+ Iron	7	0.76 ± 0.04	5.84 ± 0.48	0.16 ± 0.04	7.99 ± 0.91
Mn SOD	Control	9	27 ± 5	205 ± 34	5.78 ± 1.01	266 ± 39
	+ Iron	7	25 ± 2	189 ± 19	5.12 ± 0.34	238 ± 16
Catalase	Control	9	93 ± 10	706 ± 72	19.3 ± 2.1	966 ± 184
	+ Iron	7	186 ± 44†	1359 ± 273‡	38.0 ± 8.0†	2024 ± 589
GPx	Control	9	0.39 ± 0.06	2.88 ± 0.42	0.079 ± 0.011	23.87 ± 5.63
	+ Iron	7	0.47 ± 0.04	3.57 ± 0.37	0.096 ± 0.003	33.13 ± 9.44
G-6-PD	Control	9	21.1 ± 4.3	159 ± 33	4.4 ± 0.9	208 ± 6
	+ Iron	7	29.4 ± 8.5	221 ± 63	5.7 ± 1.6	311 ± 10

* *n*, number of litters studied. Data are expressed as U enzyme activity, except for G-6-PD expressed as mU enzyme activity.
† $p = 0.03$.
‡ $p = 0.02$.

lung wet weight $F = 6.735$, $p = 0.021$; U/mg protein $F = 6.097$, $p = 0.027$). While the mean CAT activity U/mg DNA was also double that of the control group, statistical significance was not attained ($F = 3.616$, $p = 0.078$). The addition of iron as 10^{-9} to 10^{-6} M $Fe(NO_3)_3 \cdot 9H_2O$ had no effect on CAT activity in lung homogenates of either control or study groups.

DISCUSSION

No differences were seen in body or lung weight, or lung protein and DNA content, between pups of dams fed normal diets and those receiving parenteral iron. While iron-depleted gravid rats have been reported to show retarded early fetal development (22), they show no difference in term pup weight from dams fed high iron, low iron, or control diets (1, 17).

Cross *et al.* (5) reported a mean blood contamination of perfused adult lung homogenates of 20 μl which, if we assume an average lung weight of 1.1 g, represents 18 μl/g lung wet weight. The mean contamination of fetal lung homogenates with blood was less at 8.1 ± 1.3 μl/g lung wet weight. Blood contamination of fetal lung homogenates does increase with gestational age (24), as would be expected with a developing pulmonary vascular bed. Even at term, this may be expected to be less than adult values, even after perfusion, since only ~7% of cardiac output perfuses the fetal lungs *in utero* (21). The reason for the disparity between the observed absorbance peak at 415 nm reported by Cross *et al.* (5) and our maximum of 405 nm when calculating the dithionite index is not immediately apparent, but may reflect differences in spectrophotometer calibration or, more likely, in the peak absorbances of fetal and adult hemoglobin. When we examined adult blood, there was a widening and flattening of the spectral difference peak.

Loss of iron-dependent tissue enzyme activity with iron deficiency is well recognized (22), and presumably explains the loss of lung catalase activity observed in gravid rats (13) that have reduced plasma and liver ferritin concentrations (1). A reduction of CAT activity in erythrocytes and liver catalase of iron-deficient rats has been reported (18). The CAT deficiency or iron-deficient erythrocytes is reversible (20), and we have observed (25) that lung cells cultured in an iron-free medium become reversibly acatalasic. This loss of enzyme activity is probably not due to inavailability of inorganic iron acting as a cofactor, since iron addition did not increase the specific activity of catalase. Furthermore, inorganic iron cannot be expected to reactivate or increase catalase activity *per se*, since the iron must first be inserted enzymatically to form a heme during catalase formation. The reduced activity could be due to a deficiency of a more complex iron compound (*e.g.*, ferritin) acting as a cofactor, or reduced synthesis of catalase apoprotein. It is unfortunate that blood from the pups of iron-supplemented dams was not examined for CAT activity, but even if the CAT activity of the red cells doubled, as might be expected (18), this would only account for <5% of the observed increase.

In vitro observations have demonstrated enhanced hydrogen peroxide production by hyperoxic lung tissue (12, 26). Under normoxic conditions, CAT activity may not be essential since GPx can efficiently catalyze low concentrations of hydrogen peroxide (9). However, CAT activity is important during enhanced rates of cellular hydrogen peroxide production (15), and *in vivo* hyperoxia studies have shown an increase in lung CAT activity (8, 28). While anticipated iron deficiency in human pregnancy may be countered by routine use of iron supplements in clinical practice (16), a prematurely born infant may rapidly deplete limited iron reserves (27) at a time when high oxygen concentrations are administered for pulmonary immaturity. We speculate that any resultant loss of CAT activity could enhance oxygen toxicity, and that this may be reversible with iron supplements. Iron supplementation may also potentiate hydroxyl radical production (12) from hydrogen peroxide and superoxide, or other endogenous reductants, and thus increase oxygen toxicity. The positive or negative effects of iron supplementation should be studied in prematurely delivered animals, exposed to hyperoxia, before human trials are considered.

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Cardiovascular Changes in Group B Streptococcal Sepsis in the Piglet: Response to Indomethacin and Relationship to Prostacyclin and Thromboxane A_2

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Summary

Seventeen piglets were infected with a continuous intravenous infusion of live group B β-hemolytic streptococci (GBS). Hemodynamic changes were recorded, and blood samples were drawn for measurement of thromboxane B_2 (TxB_2) (stable metabolite of thromboxane A_2) and 6-keto-$PGF_{1\alpha}$ (stable metabolite of prostacyclin). Control animals ($n = 9$) received only bacteria, while treatment animals ($n = 8$) received indomethacin, 3 mg/kg IV, 15 min after the start of the bacterial infusion. Control animals responded to the bacteria within 15 min with marked elevation in mean pulmonary artery pressure (Ppa) from 15 ± 8 to 39 ± 6 mm Hg and decline in PaO_2 from 80 ± 11 to 51 ± 6 mm Hg and cardiac output (CO) from 0.24 ± 0.07 to 0.13 ± 0.07 liters/min/kg. Mean arterial blood pressure (AoP) significantly decreased from baseline value of 95 ± 13 to 51 ± 32 mm Hg by 180 min. In animals treated with indomethacin, these changes were reversed or significantly attenuated. The hemodynamic changes were associated temporally with elevations in plasma concentrations of TxB_2 or 6-keto-$PGF_{1\alpha}$. In the first 60 min, TxB_2 levels in both groups correlated with Ppa ($r = 0.72$, $p < 0.001$) and PaO_2 ($r = -0.60$, $p < 0.001$). A strong negative correlation between TxB_2 and CO was observed over the first 180 min ($r = -0.73$, $p \leq 0.001$). There was a statistically significant correlation between AoP and 6-keto-$PGF_{1\alpha}$ concentration between 60 and 180 min ($r = -0.54$, $p < 0.002$). Indomethacin improved the hemodynamic function in this model of GBS sepsis. This improvement was related in part to inhibition of synthesis of thromboxane A_2 and prostacyclin.

Abbreviations

GBS, group B streptococci
AoP, aortic blood pressure
CO, cardiac output
Ppa, pulmonary artery pressure
Ppaw, pulmonary wedge pressure
Pra, right atrial pressure
TxB_2, thromboxane B_2

Since 1962 when Northover and Sabramanian (18) first used sodium salicylate to treat endotoxin shock in dogs, numerous investigators have documented the beneficial effects of nonsteroidal anti-inflammatory agents on hemodynamics and survival in experimental endotoxin shock (6–9, 14, 15, 20–22, 32). These studies have led to further work implicating prostaglandins and thromboxanes in the pathophysiology of endotoxin shock (1–3, 10–12). Particular attention has focused on the roles of thromboxane A_2, a vasoconstrictor and platelet-aggregating agent, and prostacyclin, a vasodilator and anti-aggregating agent.

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