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The Development of Antioxidant Enzymatic Defense in the Perinatal Rat Lung: Activities of Superoxide Dismutase, Glutathione Peroxidase, and Catalase

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ABSTRACT. This report describes the development of superoxide dismutase, glutathione peroxidase, and catalase activities in fetal (days 16-22) and neonatal (day 2 postpartum) lungs of normal rats. Each of the enzymes showed

an individual pattern of development in the perinatal period. Glutathione peroxidase activity increased by 135% ($p < 0.001$) during the last 3 days before birth, catalase activity by 105% ($p < 0.01$) during the first 2 postnatal days, and the activity of superoxide dismutase by 52% ($p < 0.05$) from gestational day 19 to 2 days after birth. Contamination by superoxide dismutase from blood in the lung samples accounted for less than 2% of the activity. In contrast, not less than 10-30% of glutathione peroxidase

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and catalase originated from the blood; and corrections were made for this source of error in each sample. (*Pediatr Res* 19: 687-691, 1985)

Abbreviations

SOD, superoxide dismutase
GSH-px, glutathione peroxidase
CAT, catalase

Untoward effects of oxygen have been claimed to contribute to respiratory disorders of the newborn. This opinion has been based largely on circumstantial evidence. The alveoli are surrounded by five times higher oxygen tension at birth than during the intrauterine development. This oxygen challenge places high demands on the ability of the lungs to counteract the negative effects of this high oxygen tension. The increase in oxygen tension is even more pronounced in premature and/or asphyxiated infants as a result of oxygen therapy. Respiratory distress is known to occur more frequently after a period of intrauterine asphyxia (1). Moreover, a high oxygen tension induces pulmonary damage similar to that in the respiratory distress syndrome (2, 3). Newborn animals, including humans, have greater antioxidative protection than mature ones (4, 5). This indicates that inborn protection against an oxidative threat has developed during fetal life or shortly after birth. Premature infants, however, may be born before such protection has become fully developed, resulting in severe damage to the lungs and possibly also to other tissues. In order to evaluate this hypothesis further, we have studied the developmental patterns of the antioxidative enzymes SOD, GSH-px, and CAT from day 16-22 of gestation and 2 days after birth in the normal rat lung.

MATERIALS AND METHODS

Female Sprague-Dawley rats with a body weight of 240-260 g (Anticimex AB, Sollentuna, Sweden) were caged with males from 17.00 to 7.00 h. The day when a sperm-containing vaginal smear was found was denoted gestational day 0. Pregnant rats that were allowed to give birth did so in the morning or in the early afternoon on day 22 of gestation. The litters consisted of 8-12 newborns. Other pregnant rats were killed by a blow on the neck on days 16-22 of gestation and the fetuses were quickly removed and decapitated before any spontaneous breathing occurred. Two-day-old suckling rats were killed by decapitation. The study included three to five litters from each of gestational days 16-22 and 2 days postpartum.

Fetal and neonatal blood samples were taken from neck vessels after decapitation, immediately hemolyzed in 10 volumes of distilled water, and quickly frozen in liquid nitrogen. The lungs were excised and trimmed of nonpulmonary tissue and then repeatedly washed in cold physiological saline, frozen in liquid nitrogen, and subsequently kept at -70°C . After thawing, the lung samples were diced with scissors, weighed, and placed in 1.2 ml of fresh, cold phosphate-buffered saline. The samples were sonicated (Sonifier Cell Disrupter, Branson Sonic Power Company, CT) for 20-30 s and chilled on ice. After removal of 200- μl samples from the lung homogenates for DNA estimation, the remaining solution was centrifuged at $8200 \times g$ for 5 min (Biofuge, Heraeus-Christ, Osterode, FRG), and the supernatant was decanted into different vials and refrozen in liquid nitrogen and stored at -70°C until assayed for its enzyme activity and hemoglobin content.

Chemicals. Rat albumin, reduced glutathione, glutathione reductase, calf thymus DNA, and t-butylperoxide were obtained from Sigma Chemical Company, St Louis, MO; bovine liver catalase and NADPH were from Boehringer Mannheim GmbH, FRG. EDTA (Titriplex III), hydrogen peroxide (H_2O_2 ; Perhydrol

30%), pyrogallol (1,2,3 benzenetriol), and diethylene triamine pentaacetic acid (Titriplex V) were purchased from Merck, Darmstadt, FRG.

Assays. The hemoglobin concentration was determined in thawed lung samples and hemolyzed blood from the same animals according to a method of Marklund (6), utilizing the peroxidase activity of hemoglobin. This method was applicable to rat hemoglobin, using rat serum instead of human and with 0.5% rat albumin as protein control. GSH-px (EC 1.11.1.9) was assayed by a slight modification of the method of Günzler *et al.* (7). The reagent solution consisted of 0.1 M Tris-HCl, pH 7.4, 0.9 mM EDTA, 0.19 mM nicotinamide adenine dinucleotide phosphate, reduced, 2.3 mM reduced glutathione, 1.2 U/ml glutathione reductase, and 0.6 mM t-butylperoxide. The latter was used as substrate instead of H_2O_2 , giving the method higher sensitivity (8). Reaction rates were determined at 340 nm and 37°C (Perkin-Elmer 555 UV-VIS spectrophotometer, Bodensee, FRG). One katal of GSH-px activity was defined as the amount of enzyme leading to the oxidation of 1 mol of nicotinamide adenine dinucleotide phosphate, reduced, per second under the conditions used. The hemolyzed blood samples were analyzed in the same way to determine the GSH-px activity in the blood. CAT (EC 1.11.1.6) activity was measured with a Clark oxygen electrode (Eschweiler, Kiel, FRG) as described by Del Rio *et al.* (9). Lung homogenate or hemolyzed blood was added to deaerated 10 mM potassium phosphate buffer, pH 7.4, with 0.1 mM EDTA and 15 mM H_2O_2 . The catalase activity was calculated from the initial rate of O_2 liberation. For the calculations a standard curve was constructed for bovine liver catalase. One unit of catalase decomposes 1 μmol of H_2O_2 per minute at 25°C , pH 7.4. SOD (EC 1.15.1.1) activity was determined by the pyrogallol method of Marklund and Marklund (10). The inhibitory effect of SOD on the autooxidation of pyrogallol (1,2,3-benzenetriol) at pH 8.20 was assayed spectrophotometrically at 420 nm and 25°C . The reaction was run in 50 mM Tris-HCl, 0.2 mM pyrogallol, 1 mM DTPA, and 0.2 mg of catalase per milliliter. One unit of SOD activity was defined as the amount of the enzyme that inhibits the pyrogallol autooxidation with 50%. DNA was measured in duplicate samples of lung homogenates as described by Kissane and Robins (11) and modified by Hinegardner (12). The fluorescence was read in a Farrand Ratio Fluorophotometer (Farrand Optical Co., Mt. Vernon, NJ) using calf thymus DNA as a standard.

Statistics. For each gestational age the mean value of three to five litters was calculated. In each litter one or, more often, two randomly chosen fetuses or newborns were investigated. Student's two-tailed *t* test was used for statistical evaluations of differences and trends.

RESULTS

In the pilot studies preceding this work it was apparent that 10-30% of the GSH-px and CAT found in the fetal lung homogenates originated from contaminating blood. Initially it was not possible to estimate the contribution of SOD activity in the blood to the activity measured in the lung samples, since the absorbance of the hemoglobin in the blood hemolysate interfered with the SOD analysis. In order to estimate the proportion of SOD activity deriving from the blood, the hemolysate from day-20-old fetuses was made hemoglobin free by a modified Tsuchihashi method (13) as described by Saik *et al.* (14). This method involves the precipitation of hemoglobin without SOD being influenced (15). With this procedure the SOD in fetal hemolysate could be determined. The contamination of the lung homogenates by blood-borne SOD was thus found to be less than 2%. Consequently the blood contributions of GSH-px and CAT were therefore subtracted from the values in the lung tissues, but no correction was made for the minute contribution of SOD from the blood.

Figures 1 and 2 demonstrate the relative importance of correc-

Fig. 1. GSH-px activity in rat lungs from gestational day 16 to day 2 postpartum (*pp*), expressed as $\mu\text{kat/g}$ lung (wet weight). The shaded area of each bar represents the GSH-px activity in the blood contained in the lung samples (means $\pm$ SE, figures within bars indicate the number of litters used).

Fig. 2. CAT activity in rat lungs from gestational day 16 to day 2 postpartum (*pp*), expressed as U/g lung (wet weight). The shaded area of each bar represents the CAT activity in blood contained in the lung samples (means $\pm$ SE, figures within bars indicate the number of litters used).

tion for the contamination of blood-borne GSH-px (Fig. 1) and CAT (Fig. 2) in the lung homogenates. As seen in Figures 1 and 2 the absolute contributions of enzyme activity from the blood remained essentially unchanged with time. The activity of GSH-px in the lung increased only slightly during early fetal life (Fig. 3). This increase was strongly augmented in the last 3 days before birth ($p < 0.001$), and on day 22 the activity was comparable to that in infants 2 days old, both when calculated per gram wet weight (Fig. 1) and when calculated per milligram of DNA (Fig. 3). As in the case of GSH-px, the CAT activity showed only a minor increase during intrauterine life. The main acceleration of CAT activity occurred after birth. Thus, the level of this enzyme was twice as high in the first 2 days of life as on day 22 of gestation ($p < 0.01$) (Fig. 4). The SOD activity showed a slight increase between gestational day 19 and day 2 postpartum ($p < 0.05$), but during the last days of intrauterine life no significant changes were seen (Fig. 5).

DISCUSSION

Only a few investigations have previously been undertaken to evaluate the development of antioxidative enzymes during fetal life in the rat lung. Yam *et al.* (16) found increases in SOD, CAT, and GSH-px activity in newborn rats compared with rats that were 2 days premature. SOD and GSH-px then increased

Fig. 3. GSH-px activity in rat lung homogenates between gestational day 16 and day 2 postpartum (*pp*), corrected for lung blood GSH-px activity and expressed in $\mu\text{kat/mg}$ DNA (means $\pm$ SE, figures within brackets indicate the number of litters used).

gradually with age, while the CAT activity after birth declined to the adult level, which was about 50% of that in neonates, confirming Roberts' previous results (17). Yoshioka *et al.* (18) reported low fetal levels of SOD activity (53%) and GSH-px activity (30%) compared with adult ones. No age-related variations were found with respect to CAT. Late gestational changes in lung antioxidant enzymes have been observed by Frank and Groseclose (19) in fetal rabbits. The lung activity of SOD and of CAT both increased by 110% and that of GSH-px by 200% during the last 3–5 days prenatally.

Our investigation was focused on the development of the antioxidant enzymes in the rat lung during the last third of intrauterine life and the period immediately after birth. We have established that the three enzymes develop in different ways. GSH-px in the lung shows a remarkable increase during the last 3 days prior to birth, and CAT is doubled in 2 days after birth. The SOD activity, on the other hand, increases slowly with time, with less age-related variation than GSH-px and CAT. These differences between the rates of development, in fetal rat lung, of the three enzymes responsible for the defense mechanism of oxygen toxicity have not been reported previously and may indicate a functional dissimilarity in their mode of action. The mechanism underlying the increase in GSH-px activity before the oxygen challenge is not known. It may be seen as a preparation for birth. Furthermore, GSH-px seems to be important for the protection against oxidant-induced pulmonary damage. After exposure of the neonatal rat lung to hyperoxia, the enzyme activity was to increase by 30% after 24 h (20). However, the process of lung maturation accelerates considerably between day 20 of gestation and birth, especially in relation to glycogen and phospholipid metabolism (21, 22). This maturation may involve metabolic pathways which form lipid hydroperoxides or H_2O_2

Fig. 4. CAT activity in rat lung homogenates between gestational day 16 and day 2 postpartum (pp), corrected for lung blood CAT activity and expressed in U/mg DNA (means $\pm$ SE, figures within brackets indicate the number of litters used).

even before birth. A concomitant increase in the intracellular levels of antioxidant enzyme therefore may occur.

The importance of CAT in the antioxidative defense is less clearly understood than that of GSH-px. The increase in CAT activity can be explained as a direct response to an augmented tissue level of H_2O_2 after birth. The generation of H_2O_2 by lung mitochondria is proportional to the surrounding oxygen tension (23). Most CAT is located in peroxisomes, but a recent report (23) would indicate that CAT may also exist in lung mitochondria, although the data are inconclusive. Under hyperoxic conditions with excess H_2O_2 formation, CAT seems to play a more important antioxidant role than under normoxic conditions, when GSH-px handles the catalysis of low concentrations of H_2O_2 more efficiently (24).

SOD seems to be less age dependent than CAT and GSH-px. It is possible that this basal level of SOD during fetal life is sufficient to protect the lung cells from superoxide radicals and that the activity will rise only under extreme hyperoxic conditions in an environment containing more than 70% oxygen (25).

While this manuscript was under preparation, Tanswell and Freeman (26) reported a similar study. They found a similar developmental pattern for GSH-px, but the increase in CAT between days 20 and 22 and just after birth observed in the present investigation was not seen in their study. Since they have subsequently shown (27) that the CAT level in prenatal rat lung is highly influenced by the iron intake of the pregnant dams, the discordance between the results may possibly be explained by differences in the nutritional status of the animals in the two studies. The very moderate perinatal increase in total SOD (about 30%) in the present study fits well with the data of Tanswell and Freeman (26) if their CuZn-SOD (copper zinc-SOD) and Mn-SOD (manganese-SOD) values are combined to yield total SOD activity. They reported that a majority of the SOD activity was

Fig. 5. SOD activity in rat lung homogenates between gestational day 19 and day 2 postpartum (pp), expressed in U/mg DNA (means $\pm$ SE of five litters).

attributed to Mn-SOD. In our hands, however, only about 30% of the total SOD value was not inhibited by cyanide, *i.e.* represented Mn-SOD activity (Gerdin E, unpublished data). These findings indicate that differences between our data and those of Tanswell and Freeman (26) are not fully explained. A definite clarification of the developmental pattern of SOD in fetal rat lung probably requires an immunological technique rather than the different bioassays used.

Our finding of different changes in the baseline levels of the three enzymes does not *a priori* indicate that premature newborns run a higher risk of suffering noxious effects of hyperoxia. The susceptibility to oxygen-induced damage is not only a function of antioxidant enzymes. Different concentrations of easily peroxidizable polyunsaturated fatty acids or easily damaged sulfhydryl enzymes, as well as different levels of low molecular weight antioxidants, such as vitamin C, vitamin E and sulfhydryl-containing substances, *e.g.* methionine or cysteine, could be equally important. Furthermore, it is not known whether the immature lung can respond to an oxygen challenge with a quick increase in enzyme levels in the same way as in the neonatal lung. An adaptation of this kind ought to be more important than the baseline levels of the oxygen detoxifying enzymes. Future studies should clarify the precise relationships between antioxidative defense mechanisms and fetal pulmonary maturation.

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The Effects of Brain Blood Flow on Brain Bilirubin Deposition in Newborn Piglets

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ABSTRACT. Since kernicteric lesions are usually found in the subcortical regions of the brain and these areas also receive the highest blood flow during asphyxia and hypercapnia, we hypothesized that increases in brain bilirubin deposition may be related to increases in brain blood flow. Fourteen piglets underwent a 3-h infusion of bilirubin to maintain total serum bilirubin at approximately 8 mg/dl, during which time blood gases, hemodynamic variables, and brain blood flow were determined. After sacrificing the

animals, regional brain bilirubin content was determined. Ten piglets underwent the same protocol; in addition, hypercapnia was induced during the last hour of study (PaCO₂ approximately 70 mm Hg). The regional brain blood flow and bilirubin deposition were significantly increased over control values ($p < 0.05$) following hypercapnia in the subcortical region and significantly so in the midbrain and cerebellum. In separate groups of control ($n = 6$) and hypercapnia ($n = 6$) piglets, ¹²⁵I-labeled albumin was infused and demonstrated that hypercapnia was not associated with increased regional brain albumin content. We conclude that hypercapnia-induced augmentation in regional brain blood flow is associated with increased deposition of unbound bilirubin. Although the causal relationship between these two observations has not been firmly established, the findings deserve future investigation to clarify the role of brain blood flow, brain bilirubin deposition, and the production of kernicterus in high risk infants. (*Pediatr Res* 19: 691-696, 1985)

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