Nitrofen-Induced Diaphragmatic Hernias in Rats: Pulmonary Antioxidant Enzyme Activities

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ABSTRACT. We developed an experimental rat model of congenital diaphragmatic hernia (CDH) to elucidate the etiology and pathogenesis of this serious congenital anomaly in humans and in particular to study the effects of a short period of artificial ventilation on the CDH lung in relation to antioxidant defense mechanisms. CDH was induced in about 60% of the offspring by maternal exposure to 2,4-dichlorophenyl-*p*-nitrophenylether (Nitrofen) during pregnancy. This herbicide resembles thyroid hormone in chemical structure. The lungs of fetal rats (d 19, 20, 21, and 22) were examined for protein and DNA content and activity of superoxide dismutase, catalase, and glutathione peroxidase (GPX). The same parameters were assessed in tracheotomized newborn rats after pressure-controlled artificial ventilation with either room air or pure oxygen during a short period of 5 h. In both CDH rats and controls, wet lung weight increased during gestation. At term, CDH rats had significantly lower mean lung weights than controls. Neither group differed in protein and DNA content per mg lung or superoxide dismutase, catalase, and GPX activity before and at birth. After artificial ventilation of neonates with air and pure oxygen, superoxide dismutase activity tended to decrease, whereas catalase activity remained virtually unchanged in the CDH lung. However, GPX activity in the CDH lung was reduced to 80% of initial activity at term after ventilation with air and to 70% with pure oxygen. The present finding of a decline in GPX activity in this animal model after a short period of artificial ventilation may indicate that the CDH rat neonate is at risk to develop oxygen-related lung damage. (*Pediatr Res* 32: 394–398, 1992)

Abbreviations

AOA, antioxidant enzyme activity
CDH, congenital diaphragmatic hernia
CuZnSOD, copper-zinc superoxide dismutase
GPX, glutathione peroxidase
NDH, rats exposed to Nitrofen without a diaphragmatic defect
SOD, superoxide dismutase

CDH is a serious congenital anomaly with unknown etiology that occurs in approximately one in 2000–5000 newborns. In contrast to other congenital abnormalities, the mortality rate of CDH has not dropped since the introduction of neonatal inten-

sive care and artificial ventilation (1). Mortality still remains at 50% as a result of pulmonary hypoplasia with or without persistent pulmonary hypertension (2). Those infants with severe respiratory compromise need maximal ventilatory support and are often subjected to high inflation pressures and high percentages of oxygen. The side effects of respiratory treatment are well known, (3) and the clinical picture of bronchopulmonary dysplasia has become one of the major sequelae of neonatal intensive care (4). Only a few reports describe defense mechanisms against oxidative stress in the human neonatal lung (5). Most experimental studies on the development of AOA have been done in small rodents (6, 7). The effect of artificial ventilation on the lung has been studied only in larger animals such as the baboon (8).

We described a model in which CDH was induced in fetal Sprague-Dawley rats by maternal exposure to a single dose of 2,4-dichlorophenyl-*p*-nitrophenylether (Nitrofen, Wako Chemicals, Neuss, Germany) during pregnancy (9, 10). The incidence of CDH in the treatment group was up to 60%. The diaphragmatic defect was mainly right-sided with severe hypoplasia of the lung as reflected by decreased lung weight/body weight ratios. No correlation was found between the size of the defect and the ipsilateral and contralateral lung weights (9). Without artificial ventilation, more than 80% of the CDH animals died within 2 h after birth as a result of severe respiratory insufficiency.

In the present study, we report 1) the activities of CuZnSOD (EC 1.15.1.1), catalase (EC 1.11.1.6), and GPX (EC 1.11.1.9) just before and at birth in the diaphragmatic hernia lung and 2) the effect of a short period of artificial ventilation on the activity of these antioxidant enzymes.

MATERIALS AND METHODS

Animal models. CDH was induced in fetal Sprague-Dawley rats by oral administration of 115 mg Nitrofen/kg body wt to the mother at d 10.5 of pregnancy as described previously (9, 10). Animals with a diaphragmatic defect showed a bilateral lung hypoplasia and smaller lung/body weight ratios compared with controls. There were no significant intragroup differences in degree of lung hypoplasia. About 40% of the fetuses did not develop CDH. The distinction between animals with CDH (group CDH) and without CDH (group NDH) could only be made after autopsy. Fetuses of pregnant rats who were not given Nitrofen served as normal controls (control group).

Fetuses were delivered by hysterotomy at d 19, 20, 21, and 22 (full term) of gestation. After determination of body weight, the fetuses were killed by intraperitoneal injection of an overdose of pentobarbital (Nembutal, Sanofi, Tindberg, The Netherlands). The presence of a diaphragmatic defect was evaluated by autopsy, after which the heart and lungs were removed *en bloc*, and the lungs were perfused with ice-cold 0.067 mol/L phosphate buffer (PBS, pH 7.8) via the pulmonary artery under constant pressure to remove the blood from the circulation. During perfusion, the

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color of the lungs changed to pale white. If part of the lungs remained pink, which indicated incomplete perfusion, the organs were discarded. The perfused lungs were then dissected, stripped of nonpulmonary tissue, weighed, frozen with liquid nitrogen, and then stored at -70°C until antioxidant activity was determined.

The effect of a short period (for practical reasons) of artificial ventilation with air or pure oxygen on pulmonary AOA was investigated in spontaneously delivered newborn rats. The presence of CDH could be suspected in Nitrofen-treated rats by observing a high ventilatory rate and persistent cyanosis with the inability to suck spontaneously (9). Artificial ventilation was performed using the technique described by Lachmann *et al.* (11). In short, the newborn rats underwent tracheostomy under pentobarbital anesthesia (35 mg/kg body wt/4 h) to allow insertion of a small cannula into the trachea. After muscle relaxation by administration of pancuronium bromide (0.1 mg/kg body wt/h), the animals were transferred to a multichambered heated body plethysmograph, which was connected to a modified Servo 900 B ventilator system (Siemens, Elema, Sweden) designed for parallel ventilation. Pressure-controlled ventilation was performed with inspiratory peak pressures of 17 cm H_2O and 2 cm H_2O of positive end expiratory pressure, respiratory rate of 40/min, and inspiratory:expiratory ratio of 1:2 according to the original reports by Lachmann *et al.* (11) on artificial ventilation in premature delivered rabbit pups. We did not increase the respiratory rate further because the rat pups showed normal thoracic excursions and remained pink. Artificial ventilation was limited to 5 h, inasmuch as CDH rats did not survive artificial ventilation with room air for longer periods. After this period, the animals were killed by an overdose of pentobarbital, after which the diaphragmatic defect in the treatment group was evaluated by autopsy. Then, the lungs were processed for AOA assay as described above. After ventilation, the lungs of control rats were processed in the same way as those of rats exposed to Nitrofen.

Biochemical analyses. All biochemical analyses were performed on separate fetal lungs. After thawing, the lungs were diluted 1:15 (wt/vol) in ice-cold PBS and homogenized with a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY) for 15 s at maximum speed according to Yam *et al.* (12). Next, the lung suspension was sonicated for 10 s on ice. In this crude suspension, concentrations of protein and DNA were estimated by Lowry's method (13), using BSA as a reference, and by a sensitive fluorimetric assay (14), respectively. To determine AOA, the crude suspensions were centrifuged at $20\,000 \times g$ for 30 min, and the pellets were discarded. The activity of the most prevailing SOD isoenzyme in the lung, CuZnSOD (15), was assayed in the supernatants by the inhibition of the xanthine (0.1 mM)-xanthine oxidase (0.025 U/mL)-catalyzed reduction of ferricytochrome *c* at pH 10.2 in the presence of 0.1 mM EDTA to chelate free copper (16, 17). Under those conditions, no spontaneous reduction of ferricytochrome *c* occurred in the presence of the supernatants under investigation. After initiating the reaction by adding xanthine oxidase, the rate of increase in absorbance at 550 nm was recorded. Various volumes of each sample were used to determine the 50% inhibition point. Only linear reaction rates were taken into account to eliminate interference of cytochrome oxidase or peroxidase with the assay. In lysates of rat erythrocytes, we found the CuZnSOD to be 9.7 times more active at pH 10.2 than at pH 7.8, which is in accordance with earlier reports (15). Because manganese SOD has the same apparent activity at pH 10.2 and pH 7.8 (18) and the results of a pilot study indicated that this isoenzyme contributes to maximally 20% of total SOD activity (data not shown), confirming earlier observations (15), the SOD assay at pH 10.2 is useful to determine changes in CuZnSOD activity. One unit of SOD activity was defined according to Crapo *et al.* (16) as the amount of enzyme needed for a 50% inhibition of the reduction of ferricytochrome *c* in the xanthine-xanthine oxidase system at

pH 7.8 using the aforementioned conversion factor of 9.7. Catalase was measured according to Bergmeyer (19); one enzyme unit (IU) was defined as the amount of enzyme that will decompose 1 μmol of H_2O_2 in 1 min at 25°C at pH 7.0 when the initial concentration of peroxide, which has a molar extinction coefficient of 41/cm at 240 nm, is 10.5 mmol/L. GPX activity was determined as previously described by Paglia and Valentine (20), who defined one unit of GPX as the amount of enzyme that converts 1 μmol of NADPH/min using a molar extinction coefficient of $6.22 \times 10^3/\text{cm}$ at 340 nm (21). To facilitate comparison and exclude differences merely based upon differences in lung weight, the activities of SOD, catalase, and GPX were all expressed as units per mg lung DNA.

Statistical analysis. In this study, lungs of four to 10 rat fetuses in each group were analyzed. The data are presented as the mean with 1 SD. After rank transformation, treatment effects were evaluated by analysis of variances (22). If a significant *F* value was found, Bonferroni's correction method for multiple comparisons was used to identify differences among the groups (23). A difference was considered statistically significant when the *p* value was 0.05 or less.

RESULTS

Lung development during gestation. Wet lung weight of normal controls increased with gestational age between d 19 and birth to 136.4 ± 21.5 mg (for time dependency, $p < 0.001$); that of CDH rats increased during gestation as well ($p = 0.007$), but the mean lung weight, amounting to 62.9 ± 10.3 mg at birth, was significantly ($0.01 < p < 0.05$) lower compared to the control and the NDH group (Table 1). The mean lung weight in the CDH and NDH rats was lower at birth than at d 21 of gestation, and also the large SD found in the control rat neonates at birth is obvious. No statistically significant differences in body weight were found between the groups during gestation (Table 1). As expressed by the significantly lower lung/body weight ratios, the CDH rats apparently developed severe lung hypoplasia *in utero* (Table 1), which affected both lungs as shown by Tenbrinck *et al.* (9). During gestation, lung protein content and DNA content expressed per mg lung weight remained virtually unchanged in all groups (Table 2); this was reflected in the protein/DNA ratio, which did not differ with time and amounted to a mean value of about 7 (Table 2). The apparent increase in lung DNA and protein within the three groups between d 21 of gestation and birth indicates that a loss of amniotic fluid from the lungs could explain the decrease in lung weight at birth.

AOA during gestation. Between d 19 of gestation and birth, the level of SOD activity remained fairly constant within the three groups (Fig. 1). Catalase activity showed a significant increase in activity between d 21 of gestation and birth in each group (control, $p = 0.007$; NDH, $p < 0.001$; CDH, $0.05 > p > 0.01$); however, there was no significant difference between the groups (Fig. 2). GPX activity steadily increased in each group with age, amounting at birth to 174, 162, and 184% of the initial GPX activity at d 19 of gestation for the control, NDH, and CDH groups, respectively (for time dependency in each group, $p < 0.001$; Fig. 3).

Effect of short period of artificial ventilation on AOA. In the control group and the NDH group subjected to a 5-h period of ventilation with room air or pure oxygen immediately after birth, SOD, catalase, and GPX activity remained at about the initial level (Table 3). Without ventilation, none of the CDH animals survived the 5 h. Under both ventilatory conditions, SOD in CDH rats showed a tendency to decrease to about 85% of the initial activity at birth (Table 3), which did not reach statistical significance ($p = 0.12$ and $p = 0.18$ for the difference between initial activity and activity after ventilation with room air and with pure oxygen, respectively). Catalase activity remained at the level reached at birth under both ventilatory conditions and resembled that of normal controls and NDH rats (Table 3). After

Table 1. Developmental changes in lung weight, body weight, and lung/body weight ratios of control rats and rats exposed to Nitrofen with and without diaphragmatic defect*

Parameter	Group†	n	Gestational age (d)			
			19	20	21	Birth
Lung weight (mg)	C	10	43.0 ± 7.1	89.6 ± 5.2	121.6 ± 7.8	136.4 ± 21.5
	NDH	7	40.8 ± 3.7	65.8 ± 30.1	137.0 ± 12.4‡	109.7 ± 8.9‡
	CDH	6	35.2 ± 5.6‡	47.6 ± 18.2‡	99.5 ± 17.1§	62.9 ± 10.3‡
Body weight (g)	C	10	1.5 ± 0.2	2.7 ± 0.3	3.9 ± 0.1	5.4 ± 0.3
	NDH	7	1.6 ± 0.1	2.6 ± 1.0	4.5 ± 0.4	4.8 ± 0.2
	CDH	6	1.5 ± 0.1	2.0 ± 0.8	4.2 ± 0.7	5.0 ± 0.4
Lung wt/body wt ratio (mg/g)	C	10	28.2 ± 2.4	33.8 ± 1.7	33.5 ± 0.9	28.1 ± 2.6
	NDH	7	25.8 ± 1.6	27.1 ± 2.7	30.9 ± 1.1	22.6 ± 1.2§
	CDH	6	24.1 ± 1.8§	26.6 ± 2.7§	24.8 ± 5.1§¶	12.2 ± 2.1§

* Data are means ± SD.

† C, control group; NDH, rats exposed to Nitrofen without diaphragmatic defect; CDH, rats exposed to Nitrofen with diaphragmatic defect.

‡ Significantly different from control, 0.01 < p < 0.05.

§ Significantly different from control, p < 0.01.

|| Significantly different from NDH group, p < 0.01.

¶ Significantly different from NDH group, 0.01 < p < 0.05.

Table 2. Developmental changes in lung protein, DNA, and protein/DNA ratio of control rats and rats exposed to Nitrofen with and without diaphragmatic defect*

Parameter	Group‡	n	Gestational age (d)			
			19	20	21	Birth
Protein (μg/mg lung wt)	C	10	71.5 ± 4.3	71.1 ± 4.7	62.8 ± 3.9	64.1 ± 7.8
	NDH	6	72.7 ± 4.3	61.7 ± 6.3	59.8 ± 6.3	65.0 ± 6.8
	CDH	6	76.9 ± 3.7	65.2 ± 6.7	63.5 ± 3.5	86.9 ± 7.1
DNA (μg/mg lung wt)	C	10	10.4 ± 0.7	8.2 ± 1.5	9.6 ± 0.7	10.3 ± 1.1
	NDH	6	11.0 ± 0.5	8.9 ± 1.4	7.5 ± 1.2	8.8 ± 0.5
	CDH	6	11.2 ± 1.0	9.7 ± 1.1	9.2 ± 0.8	12.1 ± 1.6
Protein/DNA ratio	C	10	6.9 ± 0.1	9.0 ± 1.8	6.6 ± 0.7	6.3 ± 0.5
	NDH	6	6.6 ± 0.1	7.2 ± 0.8	8.3 ± 2.0	7.4 ± 0.8
	CDH	6	6.7 ± 0.1	6.7 ± 0.1	7.0 ± 0.9	6.6 ± 0.7

* Data are means ± SD.

‡ C, Control group; NDH, rats exposed to Nitrofen without diaphragmatic defect; CDH, rats exposed to Nitrofen with diaphragmatic defect.

Fig. 1. CuZnSOD activity before and at birth expressed per mg lung DNA. Each point represents the mean + 1 SD of four to 10 lungs of normal control fetuses (O), fetuses exposed to Nitrofen without a diaphragmatic defect (Δ), and fetuses with CDH (□).

ventilation with room air, GPX activity in the CDH lung decreased to about 80% and after ventilation with pure oxygen, to almost 70% of initial activity at birth ($p = 0.05$ and $p = 0.01$, respectively) (Table 3); compared to the normal neonatal rat lung, the CDH lung shows 20% less activity under the latter condition ($p = 0.05$; Table 3).

DISCUSSION

The present study showed that before and at birth the levels of SOD, catalase, and GPX activities in the lungs of CDH and

Fig. 2. Catalase activity before and at birth expressed per mg lung DNA. Each point represents the mean + 1 SD of four to 10 lungs of normal control fetuses (O), fetuses exposed to Nitrofen without a diaphragmatic defect (Δ), and fetuses with CDH (□).

normal rats were similar. Because lung weight was significantly lower in the CDH group, total AOA, however, was reduced. This indicates that the CDH lung does not differ qualitatively from the normally developed rat lung, but only quantitatively. Furthermore, upon a short period of artificial ventilation with pure oxygen, only the CDH lung showed a small but significant decline in GPX activity.

Several studies focused on the prenatal development of pulmonary antioxidant enzymes in the rat showed that the activities

Fig. 3. GPX activity before and at birth expressed per mg lung DNA. Each point represents the mean + 1 SD of four to 10 lungs of normal control fetuses (O), fetuses exposed to Nitrofen without a diaphragmatic defect (Δ), and fetuses with CDH (□).

of total SOD, catalase, and GPX were low early in gestation and increased during fetal life (6, 7). The activity of at least one of those antioxidant enzymes is also affected by the amount of trace metals present in the food (24). Gerdin *et al.* (25) were the first to establish that the three antioxidant enzymes in the lung, *i.e.* SOD, catalase, and GPX, developed in different ways. GPX in the lung shows a remarkable increase during the last 3 d before birth, and catalase is doubled in 2 d after birth. The SOD activity, on the other hand, increases slowly with time, with less age-related variation than catalase and GPX. In the present study, we focused on the late gestational profiles (from d 19 onward) to establish whether CDH fetuses differ in AOA from normal rats. The results show that this was not the case. Within our observation period, the developmental profiles of SOD, catalase, and GPX were fairly comparable to those reported by Gerdin *et al.* (25).

In normal newborn rats, the effect of normobaric hyperoxia on the pulmonary antioxidant enzyme system has been extensively investigated (12, 26–28). We are not, however, aware of any study on the effect of artificial ventilation on the neonatal rat antioxidant enzyme system. To explore this aspect, we subjected spontaneously delivered newborn rats immediately after birth to a short period (for practical reasons) of artificial ventilation with room air or pure oxygen. The results of this study showed that under both ventilatory conditions GPX activity decreased significantly in the CDH lung, but not in the controls.

SOD activity tended to decrease in these CDH rats as well, whereas catalase activity remained at about the initial level. We do not know what caused the decrease in SOD and GPX activity of the CDH lung. At birth, the PO_2 of room air to which the neonate is exposed is about 5-fold higher than the PO_2 *in utero* (6). Under such conditions of reoxygenation, incompletely reduced forms of oxygen, *i.e.* oxygen-derived free radicals, become abundant. It is well known that SOD can be inactivated by hydrogen peroxide (29, 30) and GPX is highly susceptible to inactivation by superoxide, hydroxyl radicals, and hypochlorous acid (30–32). If the rate of degradation of these enzymes during artificial ventilation is not compensated by an increased rate of synthesis, a decline in enzyme activity can be expected.

The maturation of the pulmonary antioxidant enzyme system is under hormonal control (33–35). Thyroid hormone stimulates metabolism, generally causing an increased oxygen consumption, and stimulates tissue maturation, but is shown to depress the development of antioxidant enzymes if administered at pharmacologic doses, leading to a markedly poorer survival of pre-term rats during the first hour of life (35). It is not known whether decreased enzyme synthesis, increased enzyme inactivation, or increased enzyme turnover rate is underlying the depressed increase in AOA during gestation. Furthermore, it has been shown that in rats rendered hyperthyroid the heart showed decreased GPX activity and increased lipid peroxidation (36). Earlier studies have revealed that Nitrofen, which resembles thyroid hormone in chemical structure, is able to cross the placenta (37) and appears to have thyromimetic activity affecting the heart, the diaphragm (38, 39), and the lungs (9, 40–42). From the low lung/body weight ratio and the reduced alveolar space, as found in an earlier study (9), it can be concluded that the CDH lung in our animal model is indeed hypoplastic. At the dose of Nitrofen administered, apparently subtle differences in the exposure to this drug may exist among individual fetuses *in utero*, because about 40% of them did not develop diaphragmatic hernia. However, with respect to lung weight and lung/body weight ratio (9) (Table 1) and maybe to the GPX response under artificial ventilation as well (Table 3), those rats could be considered as intermediate between control and CDH. This led us to propose that Nitrofen as a result of its thyromimetic activity has stimulated the differentiation of the CDH neonatal lung, resembling the normal adult lung in its inability to respond to oxidative stress by an increase in AOA. Further investigations are necessary to assess the validity of this hypothesis.

Under normal steady-state conditions, antioxidant enzymes afford effective protection to tissue damage by reducing the

Table 3. Effect of short period of artificial ventilation on pulmonary SOD, catalase, and GPX activities of control rats and rats exposed to Nitrofen with and without diaphragmatic defect*

Group†	Ventilation‡	n	SOD (U/mg DNA)	Catalase (IU/mg DNA)	GPX (mU/mg DNA)
C	Birth	10	22.1 ± 2.2	142.1 ± 27.6	276.9 ± 40.5
	Air	7	20.9 ± 3.5	136.5 ± 22.7	275.8 ± 18.7
	Oxygen	8	22.8 ± 4.5	125.5 ± 13.7	271.9 ± 41.2
NDH	Birth	5	19.4 ± 4.4	146.5 ± 30.5	283.9 ± 43.6
	Air	7	20.9 ± 3.5	116.9 ± 12.4	264.1 ± 13.8
	Oxygen	9	24.6 ± 5.1	144.8 ± 31.1	255.4 ± 40.7
CDH	Birth	7	20.7 ± 4.7	124.3 ± 30.9	315.4 ± 48.4
	Air	4	17.5 ± 0.2	119.8 ± 10.1	247.9 ± 14.9§
	Oxygen	6	17.9 ± 1.9	134.0 ± 40.1	216.3 ± 54.2 ¶

* Data are means ± SD.

† C, control group; NDH, rats exposed to Nitrofen without diaphragmatic defect; CDH, rats exposed to Nitrofen with diaphragmatic defect.

‡ Neonatal rats were artificially ventilated for 5 h with air or pure oxygen. For comparison, birth values are given, because unventilated CDH neonates did not survive the first hour of life.

§ Significantly different from the same group at birth, $p = 0.05$.

|| Significantly different from the same group at birth, $p = 0.01$.

¶ Significantly different from the control group under the same ventilatory condition, $p = 0.05$.

concentration of reactive oxygen species in the lung. Superoxide is converted by SOD to hydrogen peroxide. Both catalase and GPX can effectively detoxify hydrogen peroxide, but only GPX can reduce toxic lipid hydroperoxides formed by free radical attack in the cell membrane to the less toxic hydroxy fatty acids and is therefore directly involved in the repair of the cell membrane (43). If, however, the oxygen concentration is suddenly increased, e.g. during birth or artificial ventilation, the formation of reactive oxygen species may overwhelm the pulmonary antioxidant enzyme system. A pivotal role of GPX in protection against oxidant injury is supported by the finding that tissues can tolerate loss of catalase activity during oxidative stress, but not loss of GPX activity (44, 45). In that respect, the present finding of a decrease in pulmonary GPX activity after artificial ventilation may indicate that the CDH rat neonate is at risk to develop oxygen-related lung damage.

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