Vitamin A decreases the incidence and severity of nitrofen-induced congenital diaphragmatic hernia in rats

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Thébaud, Bernard, Dick Tibboel, Caroline Rambaud, Jean-Christophe Mercier, Jacques R. Bourbon, A. Tuan Dinh-Xuan, and Stephen L. Archer. Vitamin A decreases the incidence and severity of nitrofen-induced congenital diaphragmatic hernia in rats. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L423–L429, 1999.—Congenital diaphragmatic hernia (CDH) is a major cause of refractory respiratory failure in the newborn. Pulmonary hypoplasia often limits survival. Vitamin A (Vit A) is an important signal for lung growth. We hypothesized that antenatal treatment with Vit A would stimulate lung growth and decrease mortality in experimental CDH induced in rats by ingestion of the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether). Nitrofen was administered to pregnant rats on day 12 of gestation (term 22 days). Rats were assigned to five groups: three groups received one dose of oral antenatal Vit A (15,000 IU) before (day 10), concomitant with (day 12), or after (day 14) nitrofen administration; one group received only nitrofen; and a control group received vehicle (olive oil). The incidence of CDH was markedly lower in all groups receiving Vit A (day 10, 44%; day 12, 20%; and day 14, 40%) compared with the nitrofen-treated group (84%; P < 0.05). The 72-h survival was higher in all 3 Vit A-treated groups (day 10, 40%; day 12, 58%; and day 14, 70%) compared with the nitrofen-treated group (16%; P < 0.05). Lung-to-body weight ratio and radial saccular count were significantly increased by Vit A. Antenatal treatment with Vit A lowers the incidence and severity of experimental CDH and increases lung growth and maturation.

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a cause of severe respiratory failure and persistent pulmonary hypertension of the newborn, affecting 1 in 2,000 live births (34). The outcome is related to the degree of pulmonary hypoplasia (PH) (6, 28). Notwithstanding a prenatal diagnosis by ultrasound, it is not possible to accurately predict the degree of PH and survival in these newborns (19, 42, 43). Although modern therapeutic strategies, including delayed surgery, high-frequency oscillatory ventilation, exogenous surfactant, and inhaled nitric oxide, may be beneficial in selected subjects, most newborns with hypoplastic lungs will not survive (41). As a result, the morbidity and mortality rates in CDH remain high (40–80%) (27, 33).

Antenatal therapies to favorably alter lung growth before birth have been explored to minimize PH and improve survival rates in newborns with CDH. Surgical interventions such as in utero repair of the diaphragmatic defect (1) or in utero obstruction of the trachea (PLUG technique) (5) may improve lung growth before birth. However, these techniques are invasive and limited by technical and ethical obstacles because they involve risk for both the mother and the fetus (20). Consequently, an antenatal pharmacological approach to stimulate lung growth and maturation would be appealing (38).

There is compelling evidence that retinoic acid (RA), a biologically active derivative of vitamin A (Vit A), functions as an important signal for growth and differentiation during lung development (12, 45). RA binds to specific RA receptors (RARs) that are members of the steroid-thyroid-retinoid receptor family. As early as 1953, interesting relationships between Vit A and CDH were established. Wilson et al. (44) found a high incidence of CDH in rat pups from Vit A-deficient rats. Major et al. (29) showed that markers of Vit A status (retinol and retinol-binding protein blood levels) were decreased ~50% in human babies with CDH compared with those in healthy newborns. Finally, Mendelsohn et al. (32) reported agenesis of the left lung and hypoplasia of the right lung in transgenic mice in which double deletions of RAR genes were made. In the light of these findings, the aim of our study was to test whether...
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antenatal administration of Vit A reduces the incidence of CDH and favorably alters lung growth in experimental CDH in rats. The nitrofen model described here is a well-established means of creating a nonsurgical diaphragmatic hernia with associated lung hypoplasia (13, 23, 39, 40).

MATERIALS AND METHODS

The study was approved by the Animal Care and Use Committee of the University of Cochin-Port-Royal (Paris, France).

Animal Model

Pregnant Sprague-Dawley rats were provided by Charles River (St. Aubin-les-Elbeuf, France). Observation of positive vaginal smears was considered as a proof of pregnancy (designated as day 0). The pregnant rats were transported to Faculté Cochin-Port-Royal between gestational days 6 and 8. The herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (nitrofen; 100 mg; Rohm Haas, Philadelphia, PA) was dissolved in olive oil (1 ml) and administered via an oral gastric tube on day 12 of gestation (term 22 days). Preliminary studies showed that 100 mg of nitrofen given to the mother on day 12 of gestation resulted in a 75% incidence of right-sided CDH. Mortality occurred within 24 h of birth, with most occurring immediately as previously described (39, 40).

Vit A Administration

Vit A (15,000 IU; Avibon, Rhône-Poulenc, Paris, France) was given via an oral gastric tube to the pregnant rats either before (day 10), concomitant with (day 12), or after (day 14) nitrofen. The dose of 15,000 IU was chosen according to Wilson et al. (44).

Experimental Design

Protocol 1. The first protocol was designed to study whether Vit A augments survival and reduces the incidence of CDH in nitrofen-treated animals. The animals in this protocol were randomly assigned to five groups: three groups receiving a single dose of antenatal Vit A before (day 10; nitrofen + Vit A d10), concomitant with (day 12; nitrofen + Vit A d12), or after (day 14; nitrofen + Vit A d14) nitrofen administration; one group received only nitrofen; and animals receiving only olive oil served as a control group (vehicle). There was no CDH in this vehicle group.

The rats were allowed to deliver spontaneously at term, and the newborn rats were left with their mother for feeding and warmth. The maternal rats were provided with food and water ad libitum, but otherwise no support strategies were used in the postnatal period. Survival was checked frequently until 72 h of age, at which time point all surviving animals were killed with a lethal dose of intraperitoneal pentobarbital sodium (50 mg/kg). Each animal was weighed and then dissected. The lungs and heart were removed and weighed. The presence of CDH was assessed via inspection through a median sternotomy with a dissecting microscope. The lungs, heart, and kidneys were excised and weighed. The lungs were then either frozen in liquid nitrogen for biochemical analyses or fixed in 4% paraformaldehyde for histological analyses (after prior tracheal cannulation and paraformaldehyde inflation).

Histological Studies

For the histological analyses, the trachea was cannulated and the lungs were fixed with 4% paraformaldehyde under a constant pressure of 20 cmH2O. After fixation, inflation was continued with the tracheal cannula in situ under a constant pressure of 20 cmH2O for 24 h according to Burri et al. (10). After this period, the tracheal cannula was removed and the lungs were processed for histology. The lungs were embedded in paraffin, cut into 3-µm sections, and stained with hematoxylin and eosin. The radial saccular count (RSC) was estimated according to Emery and Mithal (18) as adapted by Cooney and Thurlbeck (15), and the architecture of the pulmonary tissue was examined. Ten animals per group, six slides per animal, and ten RSCs per slide were performed. To count the saccules, an imaginary perpendicular line was drawn extending from a terminal bronchiole or a respiratory bronchiole to the closest edge of the acinus, either the pleura or a lobular septum. The number of saccular wall intercepts between the origin and the pleura is the RSC. To avoid bias, the RSC was measured on 10 segments/slide independently by two examiners who were blinded to the study groups (C. Rambaud and B. Thebaud).

Biochemical Analyses

To further evaluate lung hypoplasia, the DNA-to-protein ratio was assessed. DNA content was determined by the diphenylamine method (11). Protein content was assayed by the method of Bradford (7).

Statistical Analysis

Data are expressed as means ± SE. Intergroup differences were assessed by analysis of variance and then compared with Fisher’s probable least significant difference test with Statview (Abacus Concepts, Berkeley, CA). A P value < 0.05 was considered significant.

RESULTS

A total of 216 newborn rats (18 pregnant rats) was analyzed: 133 in the first protocol and 83 in the second protocol.

Protocol 1: Survival Rate and Occurrence of CDH

There were no overt ill effects of nitrofen on the mother. Overall, 65 (49%) rat pups survived for the predefined 72-h observation period, at which time the survivors were killed. The survival rate was significantly higher in Vit A-treated animals as shown in Fig. 1A. All hernias occurred on the right side in all study groups, and the right lobe of the liver was herniated into the right hemithorax (in whole or in part), bringing it directly in contact with the right lung. Antenatal treatment with Vit A significantly reduced the inci-
dence of CDH compared with nitrofen-treated rats that did not receive Vit A (Fig. 1B).

Protocol 2: Analyses of Lung Growth

Lung weight-to-body weight ratio. The Vit A-treated lungs were visibly larger than the nitrofen-treated lungs (Fig. 2). The lung weight-to-body weight ratio (LW/BW) was significantly higher in the vehicle- and nitrogen+Vit A-treated groups compared with that in the nitrofen-treated group (Fig. 3A). There was no difference in the LW/BW between the vehicle-treated and nitrogen+Vit A d12 groups (Fig. 3A). However, Vit A did not promote lung growth beyond normal levels in the control rats. Indeed, the LW/BW was unchanged in vehicle+Vit A d12 (0.033) and d14 (0.037) rats compared with the vehicle-treated rats (0.035; P = 0.2).

RSC. In the nitrofen-exposed rat pups, the RSC was significantly lower compared with that in the vehicle-treated group (Fig. 4). The reduced lung maturity in the nitrofen-exposed group (versus control and nitrofen+Vit A-treated groups) is evident in Fig. 2. In the nitrofen+Vit A d12 and d14 groups, the RSC was significantly increased compared with the group receiving nitrofen alone (Fig. 4). Furthermore, there was no difference in the RSC between the vehicle- and nitrogen+Vit A-treated rats (Fig. 4). Although Vit A did not increase gross lung size beyond the vehicle level, it did enhance lung maturation even in rats that were not exposed to nitrofen. For example, the RSC was significantly increased in the vehicle+Vit A d12 (4.35) and vehicle+Vit A d14 (3.7) groups compared with that in the vehicle-only group (2.96; P < 0.01 and 0.02, respectively). The histological analyses of the architecture of the lung showed advanced maturation, with thinning of the septa in the lungs from pups whose mothers received nitrofen+Vit A vs. those whose mothers received nitrofen alone.

DNA-to-protein ratio. There were no significant differences in the DNA-to-protein ratio between the vehicle-, nitrofen-, and nitrofen+Vit A-treated groups (vehicle, 0.112 ± 0.006; nitrofen, 0.117 ± 0.003; nitrofen+Vit A d12, 0.105 ± 0.003; nitrofen+Vit A d14, 0.118 ± 0.004).

The heart-to-body weight ratio was significantly lower in the nitrofen group (Fig. 3C). However, Vit A treatment increased the heart-to-body weight ratio to control levels (Fig. 3C). In contrast to the observed beneficial effects on lung and heart growth, antenatal vit A administration did not significantly change the kidney-to-body weight ratios (Fig. 3B).

DISCUSSION

The major finding of this study is that Vit A administered antenatally to pregnant rats exposed to nitrofen significantly increased survival and lung growth (expressed by the LW/BW, RSC, and DNA-to-protein ratio), whereas it decreased the incidence of CDH. The preserved ratio of DNA to protein between groups suggests that increased lung growth resulted from cell multiplication and was not due to hypertrophy of existing lung elements. Furthermore, Vit A promoted maturation of the lung architecture (Fig. 4). Importantly for possible future use in humans, Vit A was effective whether given before, concomitant with, or after nitrofen administration (Fig. 1).

The impact of a single dose of Vit A on the incidence of CDH is marked. Recent data (23, 25) suggest that CDH is a disease of impaired lung development associated with, but not necessarily caused by, a structural defect of the diaphragm. The classic mechanism of PH in CDH has been attributed to the space-occupying abdominal contents compressing the thoracic cavity (21). An alternative view of PH associated with CDH has been introduced by Iritani (23) based on experimental studies in mice fed nitrofen and supported by Kluth et al. (25). Serial study of the embryos from days 11 to 13 of
gestation demonstrated that nitrofen causes CDH by inducing PH and that normal lung development is necessary for closure of the pleural-peritoneal canal. In our study, increased lung growth induced by antenatal Vit A may have contributed to diaphragm closure. An alternative hypothesis is that Vit A may have acted directly on the mesenchymal components of the developing diaphragm, promoting its growth. RA plays important roles in the developing fetal lung by binding to specific RARs and retinoid X receptors that are members of the steroid-thyroid-retinoid receptor family. These receptors are transcription factors. The main RAR isoforms are $\alpha_1$, $\alpha_2$, $\beta_1$ $\beta_4$, $\gamma_1$, and $\gamma_2$ (32).

Mendelsohn et al. (32) suggested that RAR-$\beta_2$ plays a role in the formation of the diaphragm because they detected expression of RAR-$\beta_2$ transcripts in the anlage of the diaphragm 12.5 days postconception. Because the timing of diaphragm closure was not investigated, we can only speculate that activation of these receptors by exogenous Vit A may stimulate growth of the diaphragm, thereby closing the defect and reducing the incidence of CDH.

The fact the CDH in our study was predominantly right sided, whereas CDH in humans occurs largely on the left side, may raise the question of whether the nitrofen model is valid. No CDH model is perfect. The surgical models, for example, can only be created after the diaphragm is fully formed for technical reasons. The nitrofen model is the only one that allows study of CDH from the earliest stages (i.e., when the foregut is split into the esophagus and trachea). This is not possible with the surgical models. Although the high percentage of right-sided hernias in our study does not fully reflect the high prevalence of left-sided hernias seen in clinical situations in humans (~80%), right-sided CDH does occur in ~20% of afflicted neonates (26). Furthermore, the CDH “sideness” depends on the schedule of nitrofen administration; given on day 9 of gestation to the mother rats, nitrofen induces a mixture of left- and right-sided defects in the offspring (2), Allan and Greer (2) noted a 57% incidence in left-sided CDH when nitrofen was given on day 11 of gestation. Brandsma et al. (8) found 40% left-sided CDH when nitrofen was administered on day 11.5. We chose to administer nitrofen slightly later because lung hypoplasia is extremely severe and survival very poor if the drug is given before day 12. This later administration of nitrofen also explains the milder PH noted in the present study compared with that in previous reports (22, 37, 38). There is further evidence that the nitrofen model is relevant to the human syndrome. Specifically, the model reproduces the major abnormalities seen in human CDH (e.g., lung hypoplasia, with a reduction in the number of terminal bronchioles and the volume of alveoli, a decrease in the total size of the pulmonary vascular bed, increased thickness of the pulmonary

Fig. 2. Vit A increases lung size and maturation. A–C, top: representative examples of control (vehicle-treated) and nitrofen- and nitrofen+Vit A-treated lungs, respectively. Note that nitrofen lungs are smaller than control or nitrofen+Vit A-treated lungs. Bottom: corresponding photomicrographs of isolated lungs. Note greater development of sacculi in control and Vit A-treated lungs compared with nitrofen-treated lungs. These representative photographs illustrate retarded lung development in nitrofen-treated group. This is consistent with quantitative radial sacculary count (RSC) data in Fig. 4. Original magnification, $\times 20$. The nitrofen model is the only one that allows study of CDH from the earliest stages (i.e., when the foregut is split into the esophagus and trachea). This is not possible with the surgical models. Although the high percentage of right-sided hernias in our study does not fully reflect the high prevalence of left-sided hernias seen in clinical situations in humans (~80%), right-sided CDH does occur in ~20% of afflicted neonates (26). Furthermore, the CDH “sideness” depends on the schedule of nitrofen administration; given on day 9 of gestation to the mother rats, nitrofen induces a mixture of left- and right-sided defects in the offspring (2), Allan and Greer (2) noted a 57% incidence in left-sided CDH when nitrofen was given on day 11 of gestation. Brandsma et al. (8) found 40% left-sided CDH when nitrofen was administered on day 11.5. We chose to administer nitrofen slightly later because lung hypoplasia is extremely severe and survival very poor if the drug is given before day 12. This later administration of nitrofen also explains the milder PH noted in the present study compared with that in previous reports (22, 37, 38). There is further evidence that the nitrofen model is relevant to the human syndrome. Specifically, the model reproduces the major abnormalities seen in human CDH (e.g., lung hypoplasia, with a reduction in the number of terminal bronchioles and the volume of alveoli, a decrease in the total size of the pulmonary vascular bed, increased thickness of the pulmonary
arterial smooth muscle coat, and a decreased number of vessels per unit of lung).

In the present study, CDH rats exposed to antenatal Vit A showed an increase in lung development as assessed by LW/BW (Fig. 3A) and RSC (Fig. 4). In human and experimental CDH, there is a reduction in peripheral lung development (4, 14, 24). In our rat model, Vit A appears to have stimulated lung growth by promoting distal lung development as measured by the enhanced RSC. Consistent with this finding, Massaro and Massaro (31) recently showed that intraperitoneal administration of RA to 3-day-old rats caused a 50% increase in the number of alveoli. In the embryonic mouse lung, RA stimulated cell proliferation and branching activity through a mechanism involving epidermal growth factor (36). Other investigators (35) showed that RA stimulates proliferation of the stem cells of the alveolar epithelium.

An intriguing possibility raised by the dramatically beneficial effects of Vit A on the incidence of CDH is that nitrofen could be causing CDH by competing with RA for RARs. This possibility is supported by the observation that nitrofen binds endogenous nuclear triiodothyronine receptors (9, 30), which, similar to RARs, belong to the steroid-thyroid-retinoid receptor family.

Left heart hypoplasia has been widely documented in experimental and human CDH (3), and it seems to indicate a poor prognosis (16, 42). Furthermore, it has been shown that Vit A plays a role in normal cardiovascular development (17). In our study, nitrofen-treated rats had a significantly lower heart-to-body weight ratio compared with vehicle-treated and nitrofen + Vit A d12 and d14 animals (Fig. 3C). Therefore, Vit A may also improve the prognosis in CDH by stimulating heart growth.

In conclusion, this study showed that Vit A increases survival, decreases the incidence of CDH, and increases lung growth in nitrofen-induced CDH. These findings provide the first experimental support for the possibility that in individuals with CDH associated with severe PH and a high mortality, antenatal treatment with a pharmacological agent may prove beneficial. The molecular mechanism by which Vit A induced these beneficial effects calls for further investigation.

Fig. 3. A: comparison of lung weight-to-body weight ratio (LW/BW) between groups. LW/BW was significantly higher in vehicle-treated (n = 20 rats) and nitrofen + Vit A-treated day 12 (n = 21 rats) and day 14 (n = 21 rats) groups compared with nitrofen-treated group (n = 21 rats). Significant difference compared with vehicle-treated group: *P < 0.001; †P < 0.05. Significant difference compared with nitrofen-treated group: †P < 0.01; ‡P < 0.05. B: comparison of kidney weight-to-body weight ratio (KW/BW) between groups. Vit A did not significantly change KW/BW. *Significant difference compared with vehicle-treated group, P < 0.001. C: comparison of heart weight-to-body weight ratio (HW/BW) between groups. Vit A increased HW/BW to levels seen in vehicle-treated group. Significant difference compared with vehicle-treated group: *P < 0.001; †P < 0.01. †Significant difference compared with nitrofen-treated group, P < 0.05.

Fig. 4. Comparison of RSC between groups. In nitrofen-treated group, RSC was significantly lower compared with vehicle-treated group. In nitrofen + Vit A-treated day 12 and day 14 groups, RSC was significantly increased compared with nitrofen-treated group. Furthermore, there was no difference between vehicle- and nitrofen + Vit A-treated groups. *Significant difference compared with vehicle-treated group, P < 0.001. Significant difference compared with nitrofen-treated group: †P < 0.002; ‡P < 0.001.
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