Reductions in the incidence of nitrofen-induced diaphragmatic hernia by vitamin A and retinoic acid

Randal P. Babiuk, Bernard Thébaud, and John J. Greer

1Department of Physiology, 2Department of Pediatrics, Perinatal Research Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

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Babiuk, Randal P., Bernard Thébaud, and John J. Greer. Reductions in the incidence of nitrofen-induced diaphragmatic hernia by vitamin A and retinoic acid. Am J Physiol Lung Cell Mol Physiol 286: L970–L973; 2004. First published January 16, 2004; 10.1152/ajplung.00403.2003.—Congenital diaphragmatic hernia (CDH) is a serious medical condition in which the developing diaphragm forms incompletely, leaving a hole through which the abdominal contents can enter the thoracic space and interfere with lung growth. A perturbation of the retinoid system has been linked to the etiology of CDH. This includes findings that nitrofen, which induces CDH in rodents, inhibits the key enzyme for retinoic acid (RA) production, retinaldehyde dehydrogenase-2 (RALDH2) in vitro. Published studies indicate that antenatal vitamin A administration on gestational day (D) 12 in the nitrofen model of CDH reduced the severity and incidence of right-sided defects and lung hypoplasia. In this study, we administered nitrofen on D8, to include the induction of clinically more prevalent left-sided defects, and examined the efficacy of several vitamin A administration paradigms to gain insights into the developmental stage of susceptibility. Furthermore, we tested the hypothesis that administration of RA, the product of RALDH2 activity, is more potent than administering the substrate, vitamin A, in reducing the incidence of CDH. The incidence of CDH was reduced from ~54% (nitrofen alone) to ~32% with vitamin A treatment. The efficacy of RA treatment was very marked, with a reduction in the incidence of CDH to ~15%. Administration of vitamin A or RA on ~D10 was most effective. These data lend further support for the potential involvement of retinoid signaling pathways and the etiology of CDH and support data from in vitro studies demonstrating a nitrofen-induced suppression of RALDH2.

retinoids; congenital diaphragmatic hernia; neonate diaphragm; respiration; newborn; lung

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) occurs in ~1:3,000 live births (11). It is characterized by failure of a portion of the diaphragm muscle to form during development. This hole in the diaphragm can lead to invasion by the developing viscera of the abdominal compartment into the thoracic space, thus interfering with lung growth and development (14). Infants born with CDH at its most serious suffer from severe respiratory failure due to lung hypoplasia and pulmonary hypertension. The cost of treatment of CDH and its attendant complications is high, and, despite technological advances in equipment and surgical techniques, the mortality and morbidity remain severe (21). As a result, considerable research into the treatment and prevention of CDH and its associated problems is in progress. CDH research has benefited from the existence of an animal model of CDH. The well-established nitrofen model (8) has provided a means of producing a condition in rodents very similar to that observed in human CDH (18).

Data from the literature have suggested that the retinoid system may be involved in CDH (reviewed in Ref. 9). In 1941, Andersen (3) published data that showed that there was a 25% incidence of CDH in the litters of vitamin A-deficient rats. Diaphragmatic hernias were documented in ~15% of the offspring of double retinoic acid receptor knockout mice (16). A small clinical study showed that mothers and their infants with CDH had lower plasma retinol levels compared with the levels in mothers and healthy infants (15). A study using genetically engineered mice demonstrated a pronounced suppression of the retinoid response element by nitrofen (6). Furthermore, it has been demonstrated, using an in vitro assay, that nitrofen inhibits mammalian retinaldehyde dehydrogenase-2 (RALDH2), the enzyme catalyzing the final step in retinoic acid production (17). Collectively, these data strongly imply that the retinoid system and/or factors influenced by retinoids are candidates for involvement in the etiology of CDH.

Administration of large doses of vitamin A antenatally reduced the incidence of nitrofen-induced CDH in rats from ~80 to ~50% (23). In that study, nitrofen and vitamin A were administered at gestational age (D) 12, which produces solely right-sided defects. In this study, we examine the ability of several regimes of vitamin A administration to counter the effects of nitrofen administration on D8. This allowed us to test the efficacy of vitamin A in a paradigm that induced left-sided defects (more typically observed clinically) and to gain some insights into the developmental stage of susceptibility. Furthermore, we tested the hypothesis that administration of retinoic acid, the biologically active form of vitamin A, will be much more potent than vitamin A in reducing nitrofen-induced CDH. The rationale for the hypothesis is based on the fact that nitrofen inhibits RALDH2 in vitro. If this translates to the in vivo state, then one would predict a differential efficacy of vitamin A vs. retinoic acid administration.

MATERIALS AND METHODS

Nitrofen model of CDH. Timed-pregnant Sprague-Dawley rats (morniing of detection of sperm plug was assigned D0) were provided by the Health Sciences Laboratory Animal Services of the University of Alberta. Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether, 100 mg) was dissolved in olive oil (1 ml) by gentle sonication. Pregnant rats were anesthetized (~10 min) with halothane (1.25% in 95% O2-5% CO2), and nitrofen was administered via gavage tube in the afternoon.
on D8. All protocols were approved by the Animal Welfare Committee at the University of Alberta.

**Vitamin A administration.** Vitamin A (25,000 IU, Avibon; Rhône-Poulenc, Paris, France) was mixed with 0.5 ml of olive oil and administered via gavage tube to anesthetized pregnant rats. Animals were assigned to four groups: one group received a single dose of vitamin A on the same day as nitrofen (D8), the second was given nitrofen on D8 and vitamin A on D10, a third group received vitamin A and nitrofen on D8 with doses of vitamin A on D10 and D12. A fifth group was fed the same weight of food treated with olive oil alone to serve as controls. Fetuses were delivered by cesarean section on D16, euthanized, fixed in 4% paraformaldehyde, and dissected under a Leica dissecting microscope to determine the presence of diaphragmatic hernia.

**Statistical analysis.** Data are expressed as means ± SD. Intergroup differences in the retinol and retinoic acid protocols were determined by unpaired t-tests using the program GraphPad Prism (GraphPad Software). P values <0.05 were considered significant.

**RESULTS**

**Incidence of CDH in response to nitrofen delivery.** The incidence of diaphragmatic defects in response to nitrofen administration was 54% (n = 237). It is critical when assessing the efficacy of vitamin A and retinoic acid suppression of CDH to have an accurate measure of CDH incidence. Examination of the literature concerning nitrofen and the production of CDH shows that nitrofen induces hernias in rat fetuses from pregnant animals treated on D8 or D9 at a rate of between 41 and 69% (mean = 54.5, n = 12 studies, Table 1).

**Incidence of CDH with vitamin A treatment.** The incidence and types of hernias in response to vitamin A treatment are shown in Table 2. Treatment with vitamin A on D8 in conjunction with nitrofen did not significantly reduce the incidence of CDH. Vitamin A administered on D8 and D10; or on D8, D10, and D12; or only on D10 reduced the incidence of CDH treated with olive oil alone to serve as controls. Fetuses were delivered by cesarean section on D16, euthanized, fixed in 4% paraformaldehyde, and dissected under a Leica dissecting microscope to determine the presence of diaphragmatic hernia.

**Table 1. CDH incidence in response to nitrofen in Sprague-Dawley and Wistar rats**

<table>
<thead>
<tr>
<th>Day of Nitrofen Administration</th>
<th>Amount of Nitrofen, mg</th>
<th>Incidence of CDH, %</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8</td>
<td>100(SD)</td>
<td>54</td>
<td>current study</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>52</td>
<td>2</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>56</td>
<td>13</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>61</td>
<td>18</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>63</td>
<td>25</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>53</td>
<td>19</td>
</tr>
<tr>
<td>D9</td>
<td>100(Wistar)</td>
<td>46</td>
<td>1</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>69</td>
<td>28</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>41</td>
<td>26</td>
</tr>
<tr>
<td>D9</td>
<td>100(SD)</td>
<td>68</td>
<td>27</td>
</tr>
<tr>
<td>D9</td>
<td>100(Wistar)</td>
<td>44</td>
<td>7</td>
</tr>
<tr>
<td>D12</td>
<td>75(SD)</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>D12</td>
<td>100(SD)</td>
<td>84</td>
<td>23</td>
</tr>
</tbody>
</table>

CDH, congenital diaphragmatic hernia; SD, Sprague-Dawley; D, gestational age in days.

**Table 2. CDH incidence under the vitamin A protocol**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Left-Sided Hernias</th>
<th>Right-Sided Hernias</th>
<th>Bilateral Hernias</th>
<th>Total Hernias</th>
<th>No. of Fetuses (No. of Litters)</th>
<th>Hernias Observed, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>41(3)</td>
<td>0</td>
</tr>
<tr>
<td>Nitrofen</td>
<td>D8</td>
<td>133</td>
<td>80</td>
<td>24</td>
<td>237</td>
<td>437(31)</td>
</tr>
<tr>
<td>Retinol</td>
<td>D8</td>
<td>13</td>
<td>31</td>
<td>8</td>
<td>51</td>
<td>93(7)</td>
</tr>
<tr>
<td>Retinol</td>
<td>D8, D10</td>
<td>21</td>
<td>8</td>
<td>1</td>
<td>30</td>
<td>91(6)</td>
</tr>
<tr>
<td>Retinol</td>
<td>D8, D10, D12</td>
<td>9</td>
<td>14</td>
<td>1</td>
<td>24</td>
<td>74(5)</td>
</tr>
<tr>
<td>Retinol</td>
<td>D10</td>
<td>8</td>
<td>14</td>
<td>1</td>
<td>24</td>
<td>75(5)</td>
</tr>
</tbody>
</table>

**Fig. 1. Overall incidence of diaphragmatic hernias in response to nitrofen alone and in conjunction with vitamin A (VA) or all-trans retinoic acid (RA).** The incidence of herniation was significantly (*P < 0.05) lower with VA or RA administration compared with nitrofen alone. D, gestational age in days.
significantly. Nitrofen or nitrofen-vitamin A treatment did not adversely affect fetal survival.

Incidence of CDH with retinoic acid treatment. The incidence of types of hernias in response to all-trans retinoic acid treatment is detailed in Table 3. There was a profound reduction in the incidence of nitrofen-induced hernias in conjunction with all of the retinoic acid paradigms used. No adverse effects were observed with supplementation of the food with 1.5 mg of all-trans retinoic acid daily. The number of fetuses per animal was the same as seen in untreated animals, and no gross external malformations such as those indicative of retinoic acid teratogenesis were observed in the fetuses examined.

Figure 1 summarizes the incidence of diaphragmatic hernias and statistical differences among nitrofen, nitrofen plus vitamin A, and nitrofen plus retinoic acid groups.

DISCUSSION

In this study, we administered nitrofen on D8 to generate left-sided, right-sided, and bilateral diaphragmatic hernias (2). Coadministration of large doses of vitamin A in conjunction with nitrofen on D8 did not reduce the incidence of hernias. However, antenatal treatment with vitamin A on D8 and D10; D8, D10, and D12; or D10 did result in a statistically significant decrease in nitrofen-induced CDH (~33% incidence compared with 54% without vitamin A). This observation is consistent with the study by Yu et al. (26), who reported a trend for the reduction in the incidence of hernias induced by D9 nitrofen administration in response to administration of vitamin A on D10. The authors propose that the data did not reach statistical significance due to the small number of experimental animals used for that study. Our focus during this study was on diaphragmatic defects. However, the earlier studies by Thébaud et al. (22, 23) and Yu et al. (26), clearly demonstrate that vitamin A administration can reduce the teratogenic effects of nitrofen on the lung, thymus, and heart.

The administration of all-trans retinoic acid was markedly more effective in reducing nitrofen-induced hernias compared with those observed with vitamin A administration. Provision of RA-treated food on D8, concomitant with nitrofen exposure, decreased the incidence of hernias to ~15% compared with 54% with nitrofen treatment alone. If the nitrofen-exposed animals were provided with RA-supplemented food for 3 or 5 days following nitrofen administration, a further decrease in hernia incidence to <10% was observed.

An in vitro study has previously demonstrated that nitrofen suppresses the action of RALDH2, the key enzyme necessary for conversion of retinol to retinoic acid. This would suggest that the decreased enzyme activity could be partially countered by increasing the amount of substrate. That is consistent with the partial reduction in hernias by the administration of very large doses of vitamin A used in this and past studies (23, 26). In contrast, administration of all-trans retinoic acid bypasses the enzymatic perturbation and thus could explain the marked increase in the efficacy of hernia reduction by all-trans retinoic acid vs. vitamin A.

On the basis of the efficacy of hernia reduction of the various treatment paradigms for both vitamin A and retinoic acid, it would appear that the time period around D10 is of particular importance. Data from past studies (2, 4, 10) suggest that a defect in the mesenchymal tissue within the primordial diaphragm, the pleuropertitoneal fold (PPF), is underlying the pathogenesis of the diaphragmatic defect associated with nitrofen-induced CDH. It is known that RALDH2 and retinol-binding proteins are strongly expressed in the PPF at D13 (5, 17), when diaphragm muscle precursors and phrenic axons have reached the PPF. Given the data from the current study, the focus now is to further understand the embryogenesis of the PPF at earlier ages and the role of retinoids in its development.

ACKNOWLEDGMENTS

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GRANTS

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REFERENCES


