Mechanisms of action of the congenital diaphragmatic hernia-inducing teratogen nitrofen

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CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a serious developmental abnormality that occurs in ~1 in 2,500 live births (41). The most commonly observed type of this anomaly is the Bochdalek hernia, characterized by incomplete formation of the posterolateral diaphragm and the invasion of the thoracic cavity by abdominal viscera. The ensuing abnormal fetal breathing movements and physical constraints on the developing lungs and heart contribute to pulmonary and left ventricular hypoplasia and pulmonary hypertension (20, 35, 37). Despite improved treatment strategies, CDH continues to have significant morbidity and mortality (50, 69). Insights into the pathogenesis of the diaphragm defect have arisen from teratogenic, dietary, and genetic rodent models of Bochdalek CDH in conjunction with examination of human postmortem tissue (17). Specifically, the initial diaphragmatic defect appears to occur early (before week 5) during the embryogenesis of the amuscular component of the primordial diaphragm, the pleuropertitoneal fold. Much less is known about the etiology of CDH. However, considering the partial similarities between the pathologies observed in the teratogenic nitrofen-induced rat model and infants with CDH (1, 19, 40), the possibility that a common underlying etiology exists has received consideration.

There have been a number of hypotheses put forth to explain the teratogenic effects of nitrofen. The retinoid hypothesis suggests that abnormalities in retinoid signaling, or regulation of the retinoid pathway, lead to the development of the primary diaphragm defect and potentially to direct effects on lung development (reviewed in Ref. 33). Other studies have suggested that nitrofen alters thyroid hormone (TH) levels and thyroid hormone receptor (THR) functions, as there are stereochemical similarities between the structure of nitrofen and TH (12, 46). More recent data suggest that nitrofen is acting as an oxidizing agent and its effects may be reversed by antioxidant vitamins A, C, and E (26–31). Here, we use an array of experimental models and measurements to systematically examine the three main hypotheses of nitrofen’s mechanism of action.

METHODS

Cell culture. P19 cells (American Type Culture Collection, Manassas, VA) derived from an embryonal carcinoma induced in a C3H/He mouse were grown in MEMα (lot 1329725; Invitrogen, Burlington, ON) with 7.5% bovine calf serum, 2.5% fetal bovine serum, 100 units of penicillin, and 100 μg of streptomycin. Cells were grown in flasks coated with 0.1% gelatin under standard conditions (37°C, 5% CO2, and 95% relative humidity).

Plasmids. Plasmids were maintained and isolated using standard methods. Cells express firefly luciferase under the control of either a retinoic acid (RA) response element (RARE-Luc) or a thyroid response element (TRE-Luc), which is responsive to changes in RA or triiodothyronine (T3) levels, respectively. The renilla luciferase plasmid, pRLSV40, is constitutively expressed in transfected cells and acts as a transfection control. Full-length versions of mouse retinal aldehyde dehydrogenase (Raldh)1, 2, and 3 were kindly provided by Dr. Greg Duester (Burham Institute for Medical Research, La Jolla, CA) and subsequently subcloned into the mammalian expression vector pSG5 (Stratagene).

Luciferase assay. P19 cells were cultured in 12-well plates overnight. They were transfected with plasmids and 8 h later treated as
described below. At 24 h after treatment, the cells were washed with 1× PBS for 5 min and then lysed using Passive Lysis Buffer (Dual-Luciferase Reporter Assay System; Promega, Nepean, ON) for 20 min. The plates were wrapped in parafilm and stored at −80°C. Transfections used RARE-luciferase or TRE-luciferase and pRLSV40 and were cotransfected with either pSG5 (control), Raldh1, Raldh2, Raldh3, and CRABP II. Transfections were performed using FuGene6 transfection reagent (Roche Applied Science, Laval, QC). Treatments included nitrogen (10 nM–100 μM), 100 nM RAL, 100 nM RA, 10 μM triiodothyronine (T3), 60 μM vitamin A, 30 μM vitamin C, or 30 μM vitamin E alone or in combination as stated. After lysis, a 50-μl sample from each well was placed into a 96-well plate to determine the amount of Renilla and firefly luciferase present. Renilla luciferase (pRLSV40) and firefly luciferase (RARE- or TRE-luciferase) were detected using Stop n’ Glo buffer and luciferase assay buffer II, respectively (Dual-Luciferase Reporter Assay System, Promega). Data were collected using the Fluoroskan Ascent FL (Thermo Sci, Milford, MA) and Ascent Software Version 2.4.2 (Shetland, UK). The results were collected in Excel and normalized for transfection efficiency. An experiment, performed in triplicate, consists of transfection, treatment, and collection of results for each variable tested within the figure. A minimum of five separate experiments were combined for each graph, unless otherwise stated. Where data were combined, the values were normalized to the mean value obtained for the appropriate control. Results were analyzed using GraphPad Prism 4 (Graphpad Software, San Diego, CA). All results (means ± SD) were tested for a Gaussian distribution using a Kolmogorov-Smirnov test. For single transfection experimental data, a one-way ANOVA was performed with a Bonferroni post hoc test. In experiments in which there were multiple transfections and treatments, a two-way ANOVA was done with a Bonferroni post hoc test. RNA isolation. P19 cells were plated in 60-mm dishes coated with 0.1% gelatin and grown overnight. The next day, the media was replaced with media containing increasing concentrations of nitrogen (10 nM–10 μM), 100 nM RA, vehicle control, or media alone. After 24 h, cells were lysed, and total RNA was isolated using TRIzol reagent (Invitrogen). Briefly, cells were lysed with 2 ml of TRIzol and then mixed with chloroform. The aqueous phase containing the RNA was removed to a new tube. The RNA was precipitated with isopropanol, washed with 75% ethanol, resuspended in DEPC-treated water, and stored at −70°C.

Reverse transcription. Reverse transcription of RNA was performed using SuperScript III Reverse Transcriptase (Invitrogen). The accompanying protocol was followed. Briefly, a mixture of oligo(dT) primers, RNA, dNTP, and water was heated to 65°C for 5 min and then placed on ice for at least 1 min. Next, 5× buffer, 0.1 M DTT, and SuperScript III RT was added, and the mixture was heated to 50°C for

Fig. 1. Overview of the retinoid signaling pathway. Retinol binding protein (RBP) is the principal physiological carrier of retinol. Recent evidence suggests that STRA6, a multitransmembrane domain protein, binds to RBP and facilitates retinol uptake. Internally, retinol is bound to CRBP. Within the cytoplasm, alcohol dehydrogenase converts retinol into retinal in a reversible reaction. Retinal dehydrogenase (RALDH) then converts retinal to retinoic acid (RA) in a nonreversible, rate-limiting enzymatic reaction. Note that there are three isoforms of RALDH (RALDH1, RALDH2, and RALDH3) that are differentially distributed among tissues. Retinoic acid can either translocate to the nucleus and bind to its nuclear receptor or can remain in the cytoplasm bound to CRABP. In the nucleus, RAR (retinoic acid receptor) heterodimerizes with RXR and binds to retinoic acid response element (RARE) upstream of the promoter region of a given gene and initiates transcription. In the cytoplasm, Cyp26 enzymes preferentially break down RA bound to CRABP into polar metabolites.
45 min. The reaction was inactivated by heating to 70°C for 15 min. Concentrations of cDNA were determined using spectrophotometry. Quantitative PCR. Quantitative PCR was performed using cDNA concentrations of 50 ng/µl and TaqMan expression assays (Applied Biosystems, Streetsville, ON). Standard curves were generated for all genes to determine their efficiency. All genes examined had standard curves of 85% or higher. The expression of 18s was used as a control for the amount of cDNA present. The threshold cycle was determined and used for analysis. PCR for each sample was performed in triplicate, with the mean threshold cycle used for further comparison. The ratio of the mean value for the expression of the gene of interest vs. 18s, corrected for the efficiency, was used to determine expression levels in relation to control. The PCR was performed by iCycler (Bio-Rad Lab, Hercules, CA), and data were collected using the iCycler iQ Optical System Software version 3.1. Results were analyzed using GraphPad Prism 4. All results (means ± SD) were tested for a Gaussian distribution using a Kolmogorov-Smirnov test. A one-way ANOVA, with a Bonferroni post hoc test, was performed to determine significance.

Yeast-hormone response element assay. The Saccharomyces cerevisiae strain TPH 499 was used for transformations. Yeast was transformed with a combination of the following nuclear receptors and hormone response elements (HRE). Nuclear receptors included human THRs TRα and TRβ, mouse RA receptors RARα, β, and γ, and mouse RXRs β, and γ as described in Walfish et al. (64). The HRE were prepared as double-stranded oligonucleotides containing a single copy of a DR4 consensus sequence TRE, F2 enhancer element as a TRE, or the βRAR gene as a RARE upstream of a cytochrome c promoter (CYCl) linked to LacZ as previously described (64). The yeast transformants were isolated and grown in culture. Overnight treatment of nitrofen plus substrate and/or nuclear receptor ligand was given as indicated. Cells were harvested, washed, resuspended in Z buffer (65), and lysed with glass beads before centrifugation. Protein concentration from the supernatant was determined by the Lowry method (44) using BSA as a standard. Ten or 20 µg of protein was used for the β-gal assay, and the activities were expressed as Miller units/milligram of protein. Data were pooled from three independent experiments and calculated as mean ± SE.

STRA6 function assay. Nitrofen was dissolved in ethanol at 100 mM and diluted with serum-free media. Retinol (ROH) uptake assay using 3H-ROH-RBP and different concentrations of nitrofen for 1 h at 37°C, followed by washing with HBSS. Cells were then solubilized in 1% Triton X-100/PBS, and radioactivity incorporation was counted by scintillation counter. Experiments were done in triplicates. ROH uptake activity for each experiment was determined by subtracting the nonspecific activity of untransfected cells, which is normally ~7% of the activity of STRA6 and LRAT transfected cells.

Rat embryo isolation for retinoid measurements. Embryos were delivered via cesarean section from timed-pregnant Sprague-Dawley rats anesthetized with halothane and maintained at 37°C by radiant heat. The day in which a morning test revealed the appearance of sperm plugs was labeled as E0. Fetal ages of rats were confirmed by measuring the crown-rump length of the embryos (4). Fetuses were rinsed in distilled water; excess fluid was absorbed and placed in a 1.5-ml centrifuge tube for weighing and freezing. Exposure to light was minimized during the procedures. Samples were stored at ~80°C and shipped on dry ice to Institute of Basic Medical Sciences, University of Oslo (Norway) for HPLC analyses of ROH, RAL, and RA levels using procedures described in Sakhi et al. (57). Data are presented as means ± SE, where n is the number of times each experiment was repeated. The significance of differences was determined by Student’s t-test to compare the treatment group with the same age control group with GraphPad Prism 4.0 software. P < 0.05 was considered a significant difference in all experiments.

TH delivery. One group of animals received 100 mg of nitrofen dissolved in 1 ml of olive oil by gavage while under halothane treatment, cells were washed once with HBSS and incubated with 3 mM 3H-ROH-RBP and different concentrations of nitrofen for 1 h at 37°C, followed by washing with HBSS. Cells were then solubilized in 1% Triton X-100/PBS, and radioactivity incorporation was counted by scintillation counter. Experiments were done in triplicates. ROH uptake activity for each experiment was determined by subtracting the nonspecific activity of untransfected cells, which is normally ~7% of the activity of STRA6 and LRAT transfected cells.

Fig. 3. Analyses of nitrofen’s effect on RAR binding. Bacteria were transformed with RARα and either RXRα, β, or γ. Treatment included 100 nM cis-RA and varying concentrations of nitrofen. *P < 0.05, **P < 0.01; n = 3 for each.

Fig. 4. Response of the RARE-luciferase assay to retinoids. Relative RARE-luciferase activity increases in response to treatment with retinal and RA. Transfection with RALDH2 further increases the RARE-luciferase activity in response to retinal. *P < 0.05, **P < 0.001; n = 6.

Fig. 5. Interaction of nitrofen and RALDH2 function. Cells were transfected with either control (pSG5) or RALDH2 plasmids. Nitrofen caused a dose-dependent decrease in RARE-luciferase activity induced in the presence of RALDH2. Coadministration of retinal (100 nM) with nitrofen prevented the decrease in RARE-luciferase activity. ***P < 0.001; n = 15.
anesthesia (2.0% in 95% O₂-5% CO₂) at noon on the eighth day of gestation (E8). A second group of animals was also treated with the same dose of nitrofen on E8, as well as receiving 150 \( \mu \)g/kg \( \text{L-thyroxine (T4; Sigma)} \) dissolved in sterile saline (total vol \( \text{200 l} \)) by intraperitoneal injection from E8 until E13, spanning the critical period of diaphragm development when nitrofen is acting to cause CDH. Fetuses from both groups were harvested at E16.5 and dissected to determine the incidence of diaphragmatic hernia.

RESULTS

Retinoid signaling pathway. Figure 1 shows a simplified schematic of the retinoid signaling pathway. We designed experiments to evaluate the actions of nitrofen at several stages of the signaling cascade. The use of the P19 cell line provided an effective assay for testing the possibility that nitrofen was affecting retinoid function by modulating mRNA of key elements in the signaling cascade. Quantitative PCR was used to measure mRNA for retinoic acid receptors (\( \text{Rars} \)), cellular binding proteins, the rate-limiting enzyme for RA production, \( \text{Raldh1}, \text{Raldh2}, \text{or Raldh3} \), and the RA degradation enzyme \( \text{Cyp26A1} \). Cells were first treated with RA, and as expected from previous studies (5, 16, 24, 34, 66), there was a marked upregulation of mRNA expression for cellular RA binding protein (\( \text{Crabp2}, \text{Rarb}, \text{and Cyp26a1} \) (Fig. 2). Thus the cell assay was performing as designed. Subsequent application of nitrofen did not affect the mRNA expression of \( \text{Raldh2}, \text{Crabp2, Rara, Rarb}, \text{and Cyp26a1} \) (Fig. 2). Note that the concentration of nitrofen added to the media was equal to 0.01% of the amount given to the dam in vivo and is equivalent to the amount that reaches embryos based on \( ^{14} \text{C-tagged nitrofen bioavailability studies (45). Furthermore, past in vitro studies (14, 48)} \) have indicated that the EC\(_{50} \) for inhibiting \( \text{RALDH2} \) is \( \sim 12 \mu \text{M} \), and doses above 100 \( \mu \text{M} \) are lethal to embryos maintained in vitro.

To determine if nitrofen was interfering with binding of RA to RA receptors, we used a yeast-HRE assay (64). Bacteria were transformed with multiple combinations of \( \text{Rarx} \) and

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**Fig. 6. Interaction of nitrofen and function of other RALDH isoforms. Cells were transfected with either control (pSG5), RALDH1, RALDH2, or RALDH3 plasmids. A clear dose-dependent decrease in relative RARE-luciferase activity is seen with increasing doses of nitrofen for all 3 RALDH enzyme transfections. \( **P < 0.01, ***P < 0.001; n = 15. \)**

**Fig. 7. Retinol uptake via STRA6 transporter in the presence of nitrofen. The effect of nitrofen on retinol uptake activity of STRA6 and LRAT-transfected COS-1 cells. The activity of transfected cells without nitrofen is defined as 100%. STRA6 function persists and actually increases significantly in the presence of nitrofen. \( *P < 0.05. \)**

**Fig. 8. HPLC measurements of retinoids. Values of retinol (ROH; A), retinal (RAL; B), and RA (C) in homogenates from whole embryos of different ages after exposure to nitrofen on E9. \( *P < 0.05; n = 5–7. \) RA levels are diminished by nitrofen exposures at all ages studied.**
Rvxα, β, or γ receptors. The data for all of the combinations tested are shown in Fig. 3. In summary, there was significant inhibition of receptor binding at very high concentrations of nitrofen (100 μM), but little significant effect at lower concentrations that induce CDH without being embryonic lethal (14).

We next considered whether nitrofen was interfering with the production of RA. A dual luciferase assay system and RARE-luciferase was used as an indirect measure of RA levels in P19 cells. The first step was to demonstrate that the assay was effective for detecting changes in RA levels and responding to transfection with plasmids containing the Raldb. Data in Fig. 4 confirm the efficacy of the assay system. Note that Raldb transfection plus RAL treatment did not saturate the RA receptor’s ability to activate synthesis of luciferase. Nitrofen (10 nM-10 μM), RAL (100 nM), or RA (100 nM) did not affect the detection of luciferase or Renilla activity (Fig. 5 for nitrofen). Cells were then transfected with Raldb and treated with increasing concentrations of nitrofen. A clear dose-dependent decrease in RA levels is seen with increasing nitrofen concentrations (Fig. 5). To determine if nitrofen was a specific RALDH2 inhibitor or a more general RALDH inhibitor, cells were transfected with either Raldb1 or Raldb3. Exposure of cells to nitrofen (10 μM) caused a decrease in RARE-luciferase activity relative to control of 58%, 71%, and 59% with Raldb1, Raldb2, or Raldb3 transfection, respectively (Fig. 6).

STRA6, a cell surface receptor for ROH binding, is a recent discovery (38). Another key component of retinoid signaling, STRA6, is a member of a group of “stimulated by RA” genes. Significantly, chromosomal defects of STRA6 gene have been associated with a number of developmental anomalies including CDH (25, 53). Thus we investigated the interaction of nitrofen with this component of the retinoid signaling pathway. As illustrated in Fig. 7, nitrofen did not block ROH uptake via STRA6 function. In fact, there was an ~30% increase in uptake in the transfected COS-1 cell model.

Collectively, these data from all of the in vitro studies provided the foundation for the hypothesis that RA levels in embryos exposed to nitrofen would be significantly lower relative to control. We tested this by exposing dams to nitrofen on E9 and measuring the ROH, RAL, and RA levels of whole embryos at E11-E13 using HPLC. Figure 8 shows that at all ages, RA levels were significantly lower in embryos exposed to nitrofen. In contrast, ROH and RAL levels were not significantly lower in response to nitrofen exposure, except on days E12 and E11.5, respectively.

Nitrofen as an antioxidant. Next, the hypothesis that the perturbation of retinoid signaling by nitrofen is due to its oxidizing properties and could be rescued with administration of antioxidant vitamins was tested. Cells were transfected with Raldb2 and treated with nitrofen or a combination of nitrofen and either vitamin A, C, or E. The concentration of vitamins used was based on those reported to counter nitrofen’s effects on transcription in cultured pneumocytes (29). Data shown in Fig. 9 demonstrate that vitamin A is able to rescue the effects of nitrofen; however, vitamin C and E do not have any rescuing effect. The rescue effect of vitamin A is consistent with the previous observations that a large increase in the substrate for RALDH counters the nitrofen-induced partial inhibition of enzyme function and subsequent RA production (6). Thus, collectively, the data from the vitamin administration does not support the concept that nitrofen is acting as an oxidant to interfere with retinoid signaling.

Nitrofen as a thyromimetic. An alternative hypothesis for the teratogenic effect of nitrofen is that it is acting as a thyromimetic. The similarity in structure between T3 and nitrofen suggest that nitrofen may be acting directly on THR or by interfering with T3 binding to its receptor to alter TH signaling. To test this hypothesis, cells were transfected with both TRE-luciferase and pRLSV40 as well as either Trα or control (pSG5) plasmids. Treatment with T3 consistently resulted in a small, nonsignificant decrease in luciferase levels that became significant when cells were transfected with Trα (Fig. 10). Although this was initially unexpected, TH has been shown to have an inhibiting effect on expression of TRE regulated genes (6, 13, 42, 51, 68).

Treatment with nitrofen and/or T3 with control transfections were performed with each experiment as controls. Results show that neither treatment alone nor in combination altered the detection of either the luciferase or Renilla proteins (Fig. 10). The decrease in relative luciferase expression at 100 μM nitrofen for all test groups suggests that this treatment may be having a general effect on the cells, possibly causing cell death.
This is suggested by the decrease in Renilla expression when cells are treated with 100 μM nitrofen as well as a study showing nitrofen causes cell death in P19 cells at 100 μM after 24 h (39).

Cells transfected with Trα or pSG5 do not have changes in relative TRE-luciferase activity when exposed to nitrofen, nor does nitrofen alter relative TRE-luciferase levels when coadministered with Trα in the presence or absence of TRα (Fig. 10). These results suggest that nitrofen does not modulate TRα activity.

As an independent assay to determine if nitrofen antagonized TH binding to THR, we used the yeast-HRE assay. Bacteria were transformed with Trα in combination with Rα, β, or γ and treated with T3 (100 nM) and varying concentrations of nitrofen. Note that THR and RXR heterodimerize and bind to cis-acting thyroid HRE. There were small levels of significant inhibition under some conditions, although collectively, the data do not demonstrate a robust inhibition of THR binding by nitrofen (Fig. 11).

The final experiments were to determine if coadministration of TH and nitrofen reduced the incidence of CDH, as had been demonstrated for RA and vitamin A administration (6). As shown in Fig. 12, a dose of T4 (150 μg/kg) similar to that shown to reduce nitrofen-induced heart defects (46), did not reduce the incidence of CDH.

**DISCUSSION**

The use of the complementary models in this study allowed for a systematic evaluation of three main hypotheses regarding the pathogenesis of the nitrofen-induced model of CDH.

**Retinoids and nitrofen.** The retinoid hypothesis of the etiology of CDH arose from data derived from multiple animal studies, including: 1) induction of CDH in rats with a vitamin A-deficient diet (2, 3, 67, 70); 2) RAR double knockout mice (RARα and RARβ) produce offspring with CDH (43, 47); 3) RARE activation is decreased in mouse embryos exposed to nitrofen (14); and 4) incidence of nitrofen-induced CDH is decreased by dietary supplementation of RA (6) and to a lesser extent by vitamin A (6, 62). As outlined in Fig. 1, the retinoid signaling cascade involves multiple steps from the delivery of ROH in the blood through to the production of all-trans- and 9-cis-RA and the subsequent binding to nuclear RAR and RXR, which regulate target gene expression. The precise mechanism by which nitrofen is acting within the retinoid signaling cascade required further investigation. Quantitative PCR data showed that the expression of cellular binding protein mRNA was unaffected by nitrofen. Furthermore, the expression of RA receptor mRNA was not decreased, which is consistent with the report of Rajatapiti et al. (55) from analyses of lung tissue in both the nitrofen model and human CDH. This, however, differs from a previous report of an almost complete abolition of Rara and Rarb mRNA expression in nitrofen-exposed fetal mice (15). A change in the level of RA due to alterations in the metabolism was not implicated, as the RA degrading enzyme Cyp26a1 mRNA was not affected by nitrofen. Data from the yeast-HRE assay indicate that nitrofen is not markedly interfering with the binding of RA to RARs or RXRs. Furthermore, nitrofen did not inhibit ROH uptake via STRA6 function. The P19 cell transfection experiments showed a clear decrease in the efficacy of all isoforms of RALDH enzymes necessary to generate RA. The rescue of the nitrofen-induced inhibition by coadministration of RA further indicates a perturbation of RA as the primary underlying defect in the retinoid signaling pathway. This concept was further supported by HPLC data showing that the primary retinoid deficit was a marked decrease in RA levels, rather than ROH and RAL, in embryonic rats exposed to nitrofen. This was not
due to a suppression of *Raldh* mRNA levels and thus suggests a nitrofen-induced inhibition or RALDH function rather than expression. This is consistent with previous biochemical data showing nitrofen suppression of RALDH2 activity (48).

There is a marked reduction of embryonic RA levels during the E9-E13 period in rats indicating this as a time of particular susceptibility to perturbations of RA synthesis (58, 60). Nitrofen is typically administered late E8-E10 to induce CDH. Measurements of the nitrofen content in the embryos show that the teratogen levels are elevated within 3 h of gavage feeding and remain stable for at least 3 days, the latest time point measured (45). The timing of the nitrofen-induced diaphragmatic defect remains to be determined, but it occurs before E13.5, during the susceptible period (reviewed in Ref. 18).

It should be noted that this study determined the specific aspect of retinoid signaling affected by nitrofen. Clearly, a perturbation of any aspect of retinoid signaling or related genes could theoretically underlay the defects associated with CDH, which may well have a heterogeneous etiology. The correlation of CDH and mutations in the STRA6 gene (25, 53) is a case in point, with more candidates to follow from the emerging work on the genetic basis of CDH (see Refs. 10, 36, and 59 for review).

Antioxidant vitamins and nitrofen. While antioxidant vitamins did not rescue the nitrofen-induced perturbations of retinoid signaling in this study, they have been shown to alleviate nitrofen-induced effects in other systems. This includes partial rescue of nitrofen-induced lung hypoplasia (8, 26, 29, 49) defects in neural crest-derived tissue (26, 71), transcription in pneumocyte cell lines, and embryonic rat lung (29, 30, 71). However, there have been no reports of antioxidant treatment decreasing the incidence of CDH. Interestingly, administration of antioxidant vitamins during the period E16-E20 seems to alleviate some of the lung defects in response to administration of nitrofen on E9 (26). It is unclear whether nitrofen persists in the system for 1 wk. Thus whether vitamin therapy is specifically antagonizing nitrofen’s actions or if the vitamins are simply acting to counter damage done at an earlier stage remains unresolved. Regardless of mechanism, antioxidant vitamins as a therapeutic approach could be very significant and deserves extended investigation.

**TH and nitrofen.** TH is essential for normal vertebrate fetal development. This hormone acts to regulate gene transcription through binding its receptor, THR, which belongs to the same receptor superfamily as its binding partners, the RA receptors (reviewed in Refs. 23 and 56). Robust THR expression occurs post-E13, but there are very low levels of expression in embryos as early as E11, and thus a role for TH signaling in early diaphragm development is feasible (11, 52). Early studies reported a decrease in T3 plasma levels in dams and embryos exposed to nitrofen (45). Subsequent work has shown a decrease of T3 and T4 levels in the plasma, but importantly not in tissue levels, of nitrofen-exposed fetuses (63). In vitro binding studies showed that nitrofen is a noncompetitive antagonist of TRα and TRβ when applied at high doses (12). Terramoto et al. (61) reported a nitrofen-induced decrease expression of TRα1 and TRβ1 in rat lungs compared with controls. However, a more recent study examining both nitrofen-exposed embryonic rat lung and human lungs from CDH cases reported no change in TRα and TRβ protein or mRNA expression (55).

Thus while the data regarding TH and CDH are equivocal, they do provide a foundation for considering TH as potentially being involved in the etiology. We did not see a robust antagonizing action of nitrofen on THR except at very high doses that cause embryonic lethality and are well beyond those necessary for inducing CDH. Nor did we see a significant perturbation of TRE activation by nitrofen. Finally, we did not see a reduction in the incidence of CDH with coadministration of TH and nitrofen, similar to what can be achieved with RA rescue (6). Collectively, our data do not provide strong support for a TH relationship with the etiology of CDH. This is consistent with the lack of CDH, or other diaphragmatic defects, in several mouse models lacking functional THRs (9, 21, 22, 32).

**Summary.** Nitrofen-induced CDH has been used as an experimental model since the 1970s and has provided some important insights into the pathogenesis of this developmental anomaly. However, the fact that it is a teratogenic model affecting multiple organs suggests that caution should be taken when interpreting results in relation to clinical CDH. Furthermore, high doses used in culture can induce effects that may not be present at the lower doses sufficient to induce CDH and ensure embryo survival. Nevertheless, the diaphragmatic defects are clearly similar to the most commonly occurring Bochdalek hernias. The lungs and neural crest-derived tissues that are affected by nitrofen may in fact be independently affected in a subset of CDH cases. Thus insights into the mechanism of nitrofen’s action are likely relevant to understanding the etiology of CDH. Data from this study are most supportive of nitrofen acting to inhibit the proper synthesis of RA. It is important to note that the nitrofen model has clear similarities with the vitamin A-deficient model with regards to the pathogenesis of the diaphragm defect. Furthermore, there are similarities in the primordial diaphragm defect with the WT-1 and COUP-TFI null mouse models, and both of those genes are influenced by or interact with RA-mediated signaling (7, 54). Ultimately, it will be parallel data arising from animal models and human genetic studies that will provide insights into this clinically important problem.

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MECHANISMS OF NITROFEN ACTION