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MAIN TOPIC

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Lung hypoplasia caused by nitrofen is mediated by down-regulation of thyroid transcription factor TTF-1

Abstract Prenatal exposure to nitrofen induces lung hypoplasia and diaphragmatic hernias very similar to those in human disease, but the mechanisms are still unknown. Thyroid transcription factor 1 (TTF-1) is involved in lung ontogeny and regulation of the expression of surfactant proteins, and is likely abnormally expressed in nitrofen-induced lung hypoplasia. This study examines the effect of nitrofen on TTF-1 messenger RNA (mRNA) expression in the lungs of prenatal rat fetuses and a human lung-cell line (NCI-H441) that expresses both TTF-1 and surfactant proteins in vivo. Lungs from preterm fetuses harvested from rats with 100 mg nitrofen on gestational day 9.5 and NCI-H441 cells maintained in RPMI medium containing 10% fetal bovine serum and exposed to nitrofen for different times and concentrations were assayed for TTF-1 mRNA by northern blot analysis. mRNA for TTF-1 was decreased in nitrofen-exposed pups in comparison with controls, and exposure to nitrofen caused a dose- and time-related decrease in TTF-1 expression in H441 cell cultures. These results indicate that nitrofen downregulates TTF-1 both in vivo and in vitro. Since this interferes with lung development, it is reasonable to accept that lung hypoplasia in this model is in part due to the direct effect of the teratogen rather than to compression by the abdominal viscera herniated into the thorax. This mechanism should be explored in the clinical setting.

Key words Congenital diaphragmatic hernia · Lung hypoplasia · Nitrofen · Thyroid transcription factor 1 · Hepatocyte nuclear factor 3

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Introduction

Neonates with congenital diaphragmatic hernia (CDH) continue to have a poor prognosis. The mortality and morbidity of CDH is due to the associated severe pulmonary hypoplasia with physiological and biochemical immaturity. The fetal rat model of CDH induced by prenatal exposure to nitrofen has shown that lung hypoplasia [1] and immaturity [15] very similar to those observed in the human malformation are present in fetuses born to females treated with nitrofen, but the mechanisms remain unclear. These abnormalities are generally attributed to lung compression by the herniated viscera [11], but there are certainly other possible causes involved.

Thyroid transcription factor 1 (TTF-1) is a homeodomain-containing transcription factor (TF) expressed in two of the many structures derived from the foregut endoderm, the thyroid and the lung [13]. TTF-1 messenger RNA (mRNA) is detectable within the ventrally migrating edge of the lung bed on embryonic day (E) 10.5 in the rat (TTF-1 expression is undetectable before this stage) [13]. On E 11.5 a strong signal can be detected in both branches of the primitive bronchi, and from E 13.5 to E 15.5 TTF-1 mRNA is expressed in the bronchial epithelium. In late gestational stages (E 17.5) TTF-1 is present in epithelial cells of the bronchioli and alveolar sacs. TTF-1 is involved in both lung ontogeny and the regulation of the expression of pulmonary-specific surfactant proteins (SP-A, SP-B, and SP-C) [4, 5, 10, 18]. An essential role for TTF-1 in lung morphogenesis has been established because homozygous TTF-1 null mutant mice show dilated, saclike structures in the pleural cavity instead of normal lungs [11]. These observations suggest a possible role for TTF-1 in the pathogenesis of pulmonary hypoplasia in CDH. Using a nitrofen-induced rat model of CDH and cultures of human pneumocytes, we examined the mRNA expression of TTF-1 as an indirect marker of lung maturity.

Materials and methods

Time-mated pregnant Sprague-Dawley rats received intragastrically either 100 mg nitrofen in 1 ml olive oil (experiment group, $n = 3$) or the same volume of vehicle (control group, $n = 3$) on day 9.5 of gestation (term day 22), and their fetuses were recovered on day 21 by cesarean section. Fetal lungs were harvested and rapidly frozen for subsequent RNA extraction.

A human-lung-derived adenocarcinoma cell line, H-441, that expresses TTF-1 and pulmonary-specific markers (surfactant proteins was also used H-441 cells were obtained from the American Tissue Culture Collection (Manassas, VA) and maintained in RPMI medium containing 10% fetal bovine serum. Cells were plated 1–2 days prior to treatment with nitrofen in a time-course experiment ($n = 3$ separate experiments) for different concentrations (0.07, 0.7, and 1.5 μM) and times (24, 48, and 72 h).

Fetal lungs from the nitrofen and control groups were pooled separately ($n = 6$/group). Total RNA was isolated by the guanidine isothiocyanate/cesium chloride method. Total RNA from H-441 cells was isolated by a guanidine isothiocyanate/phenol method. Northern blot analysis was performed: 20 μg total RNA from each group was electrophoresed on formaldehyde agarose gel and transferred to a nytran membrane. Hybridization was carried out using 600 base pairs of the 3 nontranslated region TTF-1 cDNA probe. Northern blot images were analyzed quantitatively using image software and normalized with methylene blue-stained 18S ribosomal RNA bands.

Densitometric values from northern blots were normalized to 18S ribosomal RNA. All values from RNA levels in different groups were compared with controls (= 1). Relative intensities of the RNA bands were expressed as means and analyzed by Student's *t*-test for independent means. The probability $P < 0.05$ was regarded as statistically significant.

Results

The northern blot analyses for TTF-1 are shown in Figs. 1 and 2. In the nitrofen-treated animals and cells, the band intensity for TTF-1 mRNA was visibly lower than in controls. Expression of TTF-1 mRNA was markedly down-regulated in nitrofen-treated animals (70% decrease compared to controls, $P < 0.05$). Treatment of H-441 cell cultures with nitrofen caused a dose- and time-related decrease in TTF-1 expression. TTF-1 content was not significantly different from its control with low doses (0.07 μM) of nitrofen. TTF-1 expression decreased after 72 h of treatment (65% decrease over controls, $P < 0.05$) with medium doses (0.7 μM) and after 48 h of treatment (65% decrease compared to controls, $P < 0.05$) with high doses (1.5 μM) of nitrofen.

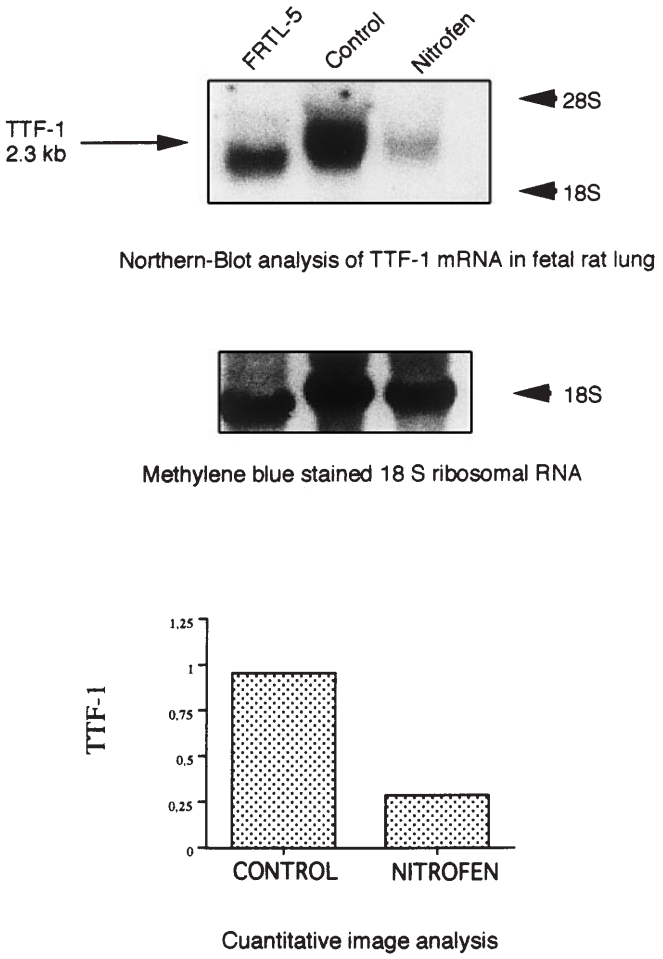

Fig. 1 Northern blot analysis of TTF-1 mRNA in fetal rat lung. Expression of TTF-1 mRNA approximately 70% decreased compared to controls

Discussion

The fetal rat model of CDH induced by prenatal exposure to nitrofen has been extensively studied, but the mechanism of the resulting pulmonary hypoplasia and immaturity remains unclear [1, 12, 15, 16]. Compression of the lung by viscera herniated into the thorax in animals with diaphragmatic defects undoubtedly plays a role, as proven by the occurrence of identical lesions in surgical models of experimental CDH in other animals [8, 14]. On the other hand, the fact that these lesions are also present, although to a lesser extent, in fetuses equally exposed to nitrofen but without CDH [1] suggests that the teratogenic action of the chemical is also directly involved.

Many TFs such as Hox, retinoid receptors, hepatocyte nuclear factors (HNF), myc, and TTF-1, a tissue-specific homeobox gene, are expressed in the lung. TFs are proteins that bind to specific sequences in the regulatory regions of genes to stimulate or inhibit transcription of target genes. In addition to binding to DNA, TFs can interact with components of the transcription complex, facilitating or impairing the ability of RNA polymerase II to initiate gene transcription. Many TFs play key roles in the control of development, regulating cell growth and differentation by modulating target genes. Two functional domains are important in these proteins, one responsible for transcription activation (transactivation domain) and the other for DNA recognition and binding (DNA binding domain).

TFs are grouped into families according to similarities in the structure of their DNA-binding domains,

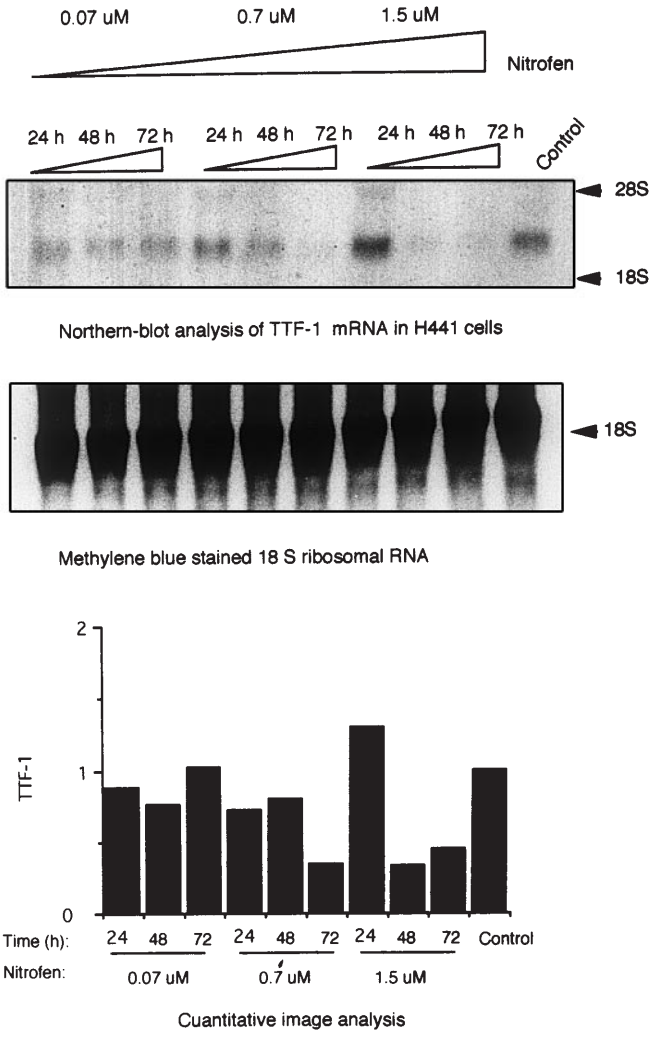

Fig. 2 Northern blot analysis of TTF-1 mRNA in human pulmonary adenocarcinoma cells. In comparison with controls, TTF-1 expression decreased approximately 65% after 72 h exposure to 0.7 μM nitrofen and 65% after 48 h exposure to 1.5 μM nitrofen

commonly called motifs. The most common motifs found in TFs are helix-turn-helix (in homeobox proteins), helix-loop-helix (c-myc, MyoD, and myogenina), winged helix HNF-3/forkhead family), leucine zipper (c-fos and c-jun), zinc finger (steroid, thyroid hormone, and retinoic acid receptor superfamily, Spl), and β-sheet (MetJ, Arc, Mnt repressors). TTF-1, also known as NKx-2.1 or T/EBP, consists of 371 amino acids and contains a homeodomain sequence with 82% identify to the *Drosophila* NK-2 homeodomain [7]. Homeodomain-containing (homeobox) proteins make up a large family of TFs that have in common a "homeodomain," a 60- amino acid helix-turn-helix motif. Generally, homeodomains recognize DNA sequences containing a 5′-TAAT-3′ or 3′-ATTA-5′ core motif.

The pattern of expression of TTF-1 transcripts resembles that shown for the surfactant proteins SP-A, SP-B, and SP-C [17]. Cotransfection assays show that TTF-1 specifically increases the activity of lung-specific gene promoters such as SP-A, SP-B and SP-C and that HNF-3 increases the activity of TTF-1 gene promoter. In addition, TTF-1 binding sites have been identified in SP-A, SP-B, and SP-C, and HNF-3 binding sites have been identified in SP-A and SP-B [4, 5, 9, 10, 18].

Some TFs may be key regulators of the development of a particular organ or set of organs, and their absence or the absence of any upstream factors may disrupt subsequent events in the cascade, as has been shown for HNF-3 and TTF-1. Homozygous mice carrying a target deletion in the HNF-3 β-gene show severe defects of the neural tube and absence of foregut structures [3]. Homozygous TTF-1 null mutants had a saclike structure in their pleural cavity, which had a rudimentary bronchial tree but no bronchioli, alveoli, or pulmonary parenchyma. The epithelium of the rudimentary bronchial tree was abnormal, consisting of hyperchromatic cells, multilayers of cells, and apparent syncytial cells. This mutant died immediately after birth of respiratory distress due to retarded lung maturation; it also lacked a thyroid and the hypothalamus was severely affected [11].

These observations, together with the development pattern of expression of the TTF-1 and HNF-3 genes, suggests that TTF-1 plays a role as a transactivating factor controlling expression of genes in the lung, but also in the development and differentiation of the lung. We tested this possibility using cultures of pneumocytes and a nitrofen-induced rat model of CDH that has shown malformations very similar to those observed in humans. A recent study [6] indicated that the expressions of PCR-amplified TTF-1 mRNA and SP-C mRNA in lungs of E 17 (2 days before term) nitrofen-treated mice were not significantly different in comparison with controls, whereas SP-A was downregulated.

We used higher doses of nitrofen (100 mg), and demostrated in fetuses recovered 1 day before term that nitrofen caused a decrease in TTF-1 mRNA expression. Moreover, we showed in vitro a dose- and time-related decrease of TTF-1 mRNA expression in cultures of H-441 cells. The intermediate dose of nitrofen used in vitro in our experiments (0.07 μM) corresponds approximately to the amount of teratogen attaining the fetal tissues of pregnant rats treated with 100 mg on the appropriate day. Lower doses did not decrease TTF-1 mRNA levels. The differences between our results and those of Coleman et al. [6] are due to the use of different doses of nitrofen, different gestational ages on fetal recovery, and different methods of mRNA assay (direct northern blot and semiquantitative PCR, respectively).

Our data indicate that nitrofen downregulates TTF-1 both in vivo and in vitro. TTF-1 is regulated by HNF-3, and both TTF-1 and HNF-3 regulate SP-B. It is reasonable to accept that nitrofen downregulates TTF-1 and SP-B by its effect on HNF-3, and that downregulation of TTF-1 contributed to lung hypoplasia. The action of nitrofen would therefore be exerted directly on the lung, causing hypoplasia that might be aggravated by compression from herniated viscera.

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