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Effects of antioxidant vitamins on molecular regulators involved in lung hypoplasia induced by nitrofen☆

Salome Gonzalez-Reyes, Leopoldo Martinez, Wenceslao Martinez-Calonge, Virginia Fernandez-Dumont, Juan A. Tovar*

Department of Pediatric Surgery, Hospital Universitario "La Paz," Paseo de la Castellana, 261, 28046 Madrid, Spain

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Abstract

Introduction: Oxidant herbicide nitrofen (2,4-dichloro-4′-nitrodiphenyl ether) induces in rat embryos congenital diaphragmatic hernia (CDH) with lung hypoplasia. The present study aims at examining whether antioxidant vitamins A, E, and C reverse the effects of the teratogen in the lungs of exposed rats and how they modify the expression of molecular regulators known to be involved in their pathogenesis.

Materials and Methods: Wet lung weight–body weight ratio, total DNA, and total protein were determined. Thyroid transcription factor 1 (TTF-1), hepatocyte nuclear factor 3β (HNF-3β), and surfactant protein B (SP-B) proteins were measured by immunoblot assay in lung homogenates from rat fetuses exposed in utero to either nitrofen 100 mg intragastrically or vehicle. The coexpression of these factors in the alveolar epithelium was demonstrated by immunohistochemistry. The effects of the addition of vitamins A, C, and E were assessed by comparison with analysis of variance.

Results: Nitrofen decreased lung weight, total DNA, and total protein. The addition of antioxidant vitamins had no effect on lung weight, but increased DNA and protein contents. TTF-1, HNF-3β, and SP-B proteins were decreased in lung homogenates of exposed rats with CDH. The addition of antioxidant vitamins nearly normalized these values.

Conclusions: The effects of nitrofen in fetal rat lungs are reversed in part by antioxidant vitamins by upregulating the expression of TTF-1, HNF-3β, and SP-B. This approach could help to develop transplacental prenatal interventions for CDH.

Congenital diaphragmatic hernia (CDH) remains a deadly malformation despite the progress achieved in neonatal intensive care. It occurs in 1:2000 to 1:4000 newborns, and the overall mortality still approaches 50% if terminations of pregnancy, abortions, stillbirths, and postnatal deaths are accounted for on a population basis [1]. The main cause of this mortality is a significant delay in pulmonary development. The ipsilateral lung and, to a lesser extent, the contralateral one are smaller, have decreased alveolar branching, thickened arterioles, and probably biochemical immaturity.

A simplistic mechanical interpretation suggests that compression of the developing lung by the herniated viscera accounts for the lesions. However, experiments in

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* Corresponding author. Tel.: +34 91 727 70 19; fax: +34 91 727 70 33.

E-mail address: jatovar.hulp@salud.madrid.org (J.A. Tovar).

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animal models and explanted embryonal lungs have shown that the parenchyma is abnormal because of a primary maldevelopment secondarily aggravated by prenatal compression [2].

Lung development starts with the outgrowth of the tracheobronchial anlage from the ventral foregut. Progressive branching and interaction of the endoderm with the surrounding mesenchyme shape the canalicular system, the alveoli, and the vascular network that will ultimately effect gas exchange. All the changes, which intervened until attaining the mature structure, are regulated by multiple organogenic cascades of genes and transcription factors and influenced by mechanical forces and growth factors [3].

Thyroid transcription factor 1 (TTF-1, also known as Nkx 2.1 or T/EBP), a member of the NK homeodomain family, and hepatocyte nuclear factor 3 (HNF-3, particularly isoforms α and β), belonging to the forkhead superfamily of transcription factors, are continuously expressed in the respiratory epithelium from the early stages of tracheobronchial branching until the end of gestation. Both transcription factors are major activators of lung-specific genes and contribute to organogenesis, differentiation, and ultimately to maturation of the terminal airway branches that will become the alveoli [4]. They are mutually regulated [5], and TTF-1 is also autoregulated [6], a fact that explains its persistent influence during the entire process of lung development [3]. Thyroid transcription factor 1 and HNF-3β coexpress in the mature type II pneumocytes that are in charge of synthesizing and secreting the lamellar bodies containing the surfactant and have a decisive influence in the regulation of the expression of surfactant proteins (SPs) A, B, and C [4]. The most important of them is SP-B, which stabilizes the phospholipids in charge of lowering superficial tension in the alveoli avoiding their collapse and facilitating gas exchange. Genetic deficiency of SP-B causes severe respiratory insufficiency in human babies as well as in newborn [7] and adult [8] mice.

The herbicide nitrofen (2,4-dichloro-4′-nitrodiphenyl ether) administered to pregnant rats or mice on appropriate embryological windows causes CDH with its associated malformations [9-11]. Hypoplasia of the lungs with decreased branching, vascular lesions, and some elements of biochemical dysmaturation have been observed in these rats [9,12].

The mechanism of action of nitrofen is only partially known. Several indications suggest that intracellular oxidative stress is one of the mechanisms involved [13].

It has been shown that prenatally administered vitamins A [14,15] and E [13,16] are beneficial for the lungs of animals with CDH, and it is likely that if their antioxidant properties contribute to their positive effects, other agents such as vitamin C could have similar effects through the same regulatory mechanisms. The present study examines the effects of prenatally administered antioxidant vitamins A, E, and C on the lung hypoplasia of CDH and on the expression of both the developmental regulators TTF-1 and HNF-3β and SP-B in rats exposed to nitrofen.

1. Materials and methods

1.1. Experimental design

The effects of antioxidant vitamins on the lungs of nitrofen-exposed fetuses with CDH were assessed in vivo by measuring lung weight, lung cell mass (DNA and protein), and expression of the proteins TTF-1, HNF3-β, and SP-B (immunohistochemistry and Western blot).

1.2. Animal experiments

Female 250-g Sprague-Dawley rats were mated with fertile male rats overnight, and vaginal smears were examined under light microscope 12 hours later. The presence of sperm signaled day 0 of gestation. Nitrofen (100 mg in 1 mL of olive oil) was given intragastrically on day 9.5 of gestation, and the animals were randomly divided into 8 groups of 6 rats: nitrofen, nitrofen + vitamin A, nitrofen + vitamin E, nitrofen + vitamin C, vitamin A only, vitamin C only, and vitamin E only. A control group in which only vehicle was administered was included. Vitamins A (Auxina Masiva, Alcala Farma, Madrid, Spain; total, 15,000 IU) and E (Ephynal Roche, Madrid, Spain; total, 150 IU) were given intragastrically in 4 doses on days 16 to 20. Vitamin C (Acido Ascorbico Roche; total, 150 IU) was given intraperitoneally on the same days. The offsprings were recovered on day 21 (term = 22nd day) to avoid cannibalism, and the fetuses were weighed and examined internally for the presence of CDH. The lungs were dissected, then were weighed and frozen at -80°C for further studies. In the 8 groups, 2 or 3 fetuses from each rat were used for each set of studies (DNA and proteins, immunohistochemistry, and Western blot) for a total of 321 pups. Only the lungs of fetuses with CDH from the experimental groups were investigated because in previous studies we demonstrated that the lung [12] and other lesions [10] were present as well in exposed animals without diaphragmatic defects, although they were less severe than those of CDH animals [17]. It was judged inappropriate to use an additional group of animals once acknowledged that the CDH lungs were developmentally abnormal and not only compressed.

1.3. Histologic and immunohistochemical techniques

The lungs were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Other sections were immunostained with anti–TTF-1 (monoclonal mouse TTF-1, Dako, Madrid, Spain), anti–HNF-3β (HNF-3β, Quimigranel, Madrid, Spain), and anti–SP-B (SP-B, Quimigranel) antibodies. They were incubated at 1:100 concentration overnight and counterstained with methyl green.