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Trichloroethylene perturbs HNF4a expression and activity in the developing chick heart

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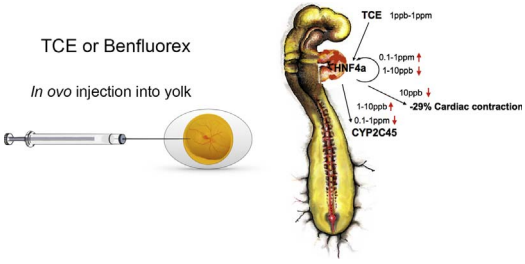
GRAPHICAL ABSTRACT



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ABSTRACT

Exposure to trichloroethylene (TCE) is linked to formation of congenital heart defects in humans and animals. Prior interactome analysis identified the transcription factor, Hepatocyte Nuclear Factor 4 alpha (HNF4a), as a potential target of TCE exposure. As a role for HNF4a is unknown in the heart, we examined developing avian hearts for HNF4a expression and for sensitivity to TCE and the HNF4a agonist, Benfluorex. *In vitro* analysis using a HNF4a reporter construct showed both TCE and HFN4a to be antagonists of HNF4a-mediated transcription at the concentrations tested. HNF4a mRNA is expressed transiently in the embryonic heart during valve formation and cardiac development. Embryos were examined for altered gene expression in the presence of TCE or Benfluorex. TCE altered expression of selected mRNAs including HNF4a, TRAF6 and CYP2C45. There was a transition between inhibition and induction of marker gene expression in embryos as TCE concentration increased. Benfluorex was largely inhibitory to selected markers. Echocardiography of exposed embryos showed reduced cardiac function with both TCE and Benfluorex. Cardiac contraction was reduced by 29% and 23%, respectively at 10 ppb. The effects of TCE and Benfluorex on autocrine regulation of HNF4a, selected markers and cardiac function argue for a functional interaction of TCE and HNF4a. Further, the dose-sensitive shift between inhibition and induction of marker expression may explain the nonmonotonic-like dose response observed with TCE exposure in the heart.

1. Introduction

Trichloroethylene (TCE) is an industrial degreasing solvent and a substrate used in chemical manufacturing. It is identified as a cardiac teratogen and carcinogen by the EPA and has a maximum contaminant level (MCL) of 5 ppb in water (Environmental Protection Agency, Chiu et al., 2006). The volatile nature of TCE allows it to easily be dispersed into the environment at large, often through the air and the

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groundwater supply. TCE was first associated with congenital heart defects (CHDs) following a water system exposure that occurred in Tucson, AZ where exposure at levels up to 270 ppb (parts per billion) produced an odds ratio of CHDs between 2.5 and 3 (Bove et al., 2002; Goldberg et al., 1990). Other studies in Madison, WI and Endicott, NY related to vapor exposure supported the results found in Tucson, AZ but the overall, epidemiological data are limited (Forand et al., 2012; Yauck et al., 2004).

Chick, mouse and rat models were used to explore the mechanistic effects of TCE on fetal cardiac formation and gene expression. Studies showed that TCE inhibits epithelial mesenchymal cell transformation in embryonic heart tissue in an *in vitro* assay at 50–250 ppm (parts per million) (Boyer et al., 2000). Abnormal cardiac gene expression, including molecules involved in calcium homeostasis and several cytochrome P450s, was observed by exposures with a rat cell line *in vitro*, in mouse maternal drinking water or injection *in ovo* of chick embryos (Caldwell et al., 2008; Collier et al., 2003; Makwana et al., 2010; Selmin et al., 2008). TCE exposure is associated with significantly increased incidence of ventricular septal defects in chick embryos (Drake et al., 2006a,b; Rufer et al., 2010). An EPA IRIS assessment stated that "weight-of-evidence analysis of epidemiological, toxicological, *in vitro*, *in ovo*, and mechanistic/AOP data concluded that TCE has the potential to cause cardiac defects in humans when exposure occurs at sufficient doses during a sensitive window of fetal development" (Makris et al., 2016).

TCE has a poorly understood but nonmonotonic-like dose curve. Low doses of TCE can produce greater changes in gene expression and function in cells and embryos compared to higher doses (Caldwell et al., 2008; Drake et al., 2006b; Makwana et al., 2013, 2010; Mishima et al., 2006). H9C2 myoblasts show a greater decrease in calcium homoeostasis at 10 ppb than they do at 100 ppb and 10 ppm doses (Caldwell et al., 2008). Epithelial-mesenchymal transition mediates the formation of cardiac valve tissues in the embryonic heart (Person et al., 2005). Mesenchymal cell formation is inhibited at higher doses of TCE (50–250 ppm) while mesenchymal cell proliferation is stimulated at 80 ppb (Mishima et al., 2006). Other work has shown that markers of blood flow show more change at 8 ppb than 800 ppb (Drake et al., 2006b; Makwana et al., 2010). It was suggested that low levels of TCE might be insufficient to induce detoxification, producing a greater concentration of TCE or an active metabolite in the tissue. However, CYP 2H1 expression was higher at the lower dose while CYP 1A4 showed higher expression at the greater dose (Makwana et al., 2013). While it is unclear which cytochromes in the chick metabolize TCE, there appears to be some cytochrome P450 enzyme induction at the lowest tested exposure.

Microarray analysis of chick embryos treated with 8 ppb TCE, *in ovo*, identified approximately 4000 genes significantly altered by TCE exposure. Among these genes, approximately 1400 were linked to genes identifiable in an interactome database (Selmin et al., 2014). Within this TCE interactome, Hepatocyte Nuclear Factor 4 alpha (HNF4a) was the most interconnected molecule as it was linked directly or indirectly to approximately 75% of the nodes (Selmin et al., 2014). HNF4a is a master transcription factor associated with the liver and not identified with the developing heart, but Western Blot and PCR data show that it can be found in the early chick heart and in cultured rat cardiomyocytes (Selmin et al., 2014).

We hypothesize that HNF4a is an early and significant target of TCE-mediated cardiac teratogenicity. To explore the hypothesis, we first tested the HNF4a-expressing HepG2 cell line to examine regulation of a transcriptional reporter by TCE and its reported agonist, Benfluorex (Lee et al., 2013). The data show that both TCE and Benfluorex inhibit HNF4a-mediated transcription *in vitro* at the doses examined. We then explored HNF4a expression during chick heart development. Quantitative PCR analysis showed that HNF4a is expressed in the developing heart during the developmental window that coincides with the published interval of sensitivity to TCE exposure (Drake et al., 2006b). Chick embryos were exposed, *in ovo,* to examine effects of TCE and Benfluorex on expression of identified targets of HNF4a and/or TCE in the heart. The data identify HNF4a, TRAF6 and CYP2C45 as markers with altered gene expression when exposed to both Benfluorex and TCE. Consistent with a nonmonotonic-like response, TCE is an inhibitor of gene expression at doses of 10 ppb or lower and an inducer of expression at doses of 100 ppb or greater. Finally, as myocyte contraction is perturbed by TCE exposure (Makwana et al., 2010), we compared embryonic heart function after low-dose TCE and Benfluorex exposure. Echocardiographic analysis showed similar decreases in cardiac contraction with either Benfluorex or TCE at 10 ppb. These data support the temporal teratogenicity of TCE in the heart during the period of cardiac myocyte and valve formation. The dose response data suggest that a basis for the nonmonotonic-like response in the developing heart may lie in the shift between inhibition or induction of gene expression by TCE.

2. Materials and methods

2.1. Reporter analysis with HepG2 cells

Human HepG2 cells (ATCC, HB-8065) were grown in DMEM media with 10% bovine serum and 1% pen/strep. Cells were incubated with either Benfluorex Hydrochloride (5 μM) (sc-291931, Santa Cruz Biotechnology) or Trichloroethylene (Cas # 70-01-6, Sigma, Aldrich). For measurement of HNF4a-mediated transcription, HepG2 cells were transfected using the Cignal HNF4a reporter kit (CCS-3039L, Qiagen) with Attractene transfection reagent (Qiagen). This kit quantifies up or down regulation of an HNF4a-responsive luciferase construct encoding firefly luciferase under control of a minimal CMV promoter and tandem repeats of the HNF4a transcriptional response element. Expression is normalized to a co-transfected constitutively active Renilla luciferase reporter. Cells were exposed to the indicated final concentrations in multiwall plates for 18 h before collection in Cell Culture Lysis Reagent (Promega, #E1531). Equal aliquots of cell lysate were mixed with Luciferase Assay Reagent (Promega, #E1500) and measured in a luminometer.

2.2. Embryos for gene expression analysis

Fertilized chick eggs were obtained from MacEntire Eggs, San Diego, California and were incubated to Hamilton and Hamburger stages (Hamburger and Hamilton, 1992) 15–26 at 37.5 °C as needed. After incubation to appropriate stages, hearts were dissected from the embryos and pooled by stages for extraction. Each pool was from 2 to 25 hearts (larger numbers were used for stages 15–18) and mean levels were calculated from 3 independent pools for each stage. RNA was collected for analysis using E.Z.N.A. Total RNA Kit 1 (R6834-02, Omega Bio-Tek). Chicken embryos, at the stages used here, are exempt from institutional, NIH and AALAC oversight.

2.3. In ovo injection

Chick embryos were incubated to Hamilton and Hamburger stage 13 before being exposed to either Trichloroethylene (Cas # 70-01-6, Sigma, Aldrich) or Benfluorex Hydrochloride (sc-291931B, Santa Cruz Biotechnology). 1x Tyrode's solution was used as the control and to dilute the chemicals. The method of exposure was a single *in ovo* injection with a 250 μL syringe (370166, Hamilton).

For injection, the eggs were turned on their side and a small hole was made with an 18 G needle (305196, BD Biosciences). 50 μL of solution was injected into the yolk of the egg. The exposure dose was either: 1 ppb, 5 ppb, 10 ppb, 100 ppb, 1 ppm of Benfluorex Hydrochloride or Trichloroethylene. The hole was covered with tape and the egg was reoriented and incubated until it reached stage 17. At HH stage 17, the heart was dissected from the chick embryo and RNA was collected for analysis.