Trichloroethylene Disrupts Cardiac Gene Expression and Calcium Homeostasis in Rat Myocytes

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We have been investigating the molecular mechanisms by which trichloroethylene (TCE) might induce cardiac malformations in the embryonic heart. Previous results indicated that TCE disrupted expression of genes encoding proteins involved in regulation of intracellular Ca\(^{2+}\), \([\text{Ca}^{2+}]_i\), in cardiac cells, including ryanodine receptor isof orm 2 (Ryr2), and sarcoendoplasmatic reticulum Ca\(^{2+}\) ATPase, Serca2a. These observations are important in light of the notion that altered cardiac contractility can produce morphological defects. The hypothesis tested in this study is that the TCE-induced changes in gene expression of Ca\(^{2+}\)-associated proteins resulted in altered Ca\(^{2+}\) flux regulation. We used real-time PCR and digital imaging microscopy to characterize effects of various doses of TCE on gene expression and Ca\(^{2+}\) response to vasopressin (VP) in rat cardiac H9c2 myocytes. We observed a reduction in Serca2a and Ryr2 expression at 12 and 48 h after exposure to TCE. In addition, we found significant differences in Ca\(^{2+}\) response to VP in cells treated with TCE doses as low as 10 parts per billion. Taken all together, our data strongly indicate that exposure to TCE disrupts the ability of myocytes to regulate cellular Ca\(^{2+}\) fluxes. Perturbation of calcium signaling alters cardiac cell physiology and signal transduction and may hint to morphogenetic consequences in the context of heart development. These results point to a novel area of TCE biology and, if confirmed in vivo, may help to explain the apparent cardio-specific toxicity of TCE exposure in the rodent embryo.

Key Words: cardiac development; environmental toxicants; trichloroethylene; calcium flux.

Heart defects are the most common birth defect-related cause of infant death in the United States with nearly one-fifth of infant deaths annually (CDC, 1998; NCHS, 2004). There are multiple lines of evidence that point to environmental factors as possible causes of congenital heart malformations. Among these are chlorinated hydrocarbons, including trichloroethylene (TCE) which has been suggested as a cardiac teratogen in humans and animal models (Bove et al., 2002; Goldberg et al., 1990; Johnson et al., 1998; Loeber et al., 1988). Recent review articles (Hardin et al., 2005; Watson et al., 2006) criticized some of the epidemiological and rodent studies. Goldberg et al. (1990) studies were faulted for the selection of controls and patients, but a recent independent re-evaluation of the data largely supported the findings (Bove et al., 2002). Criticisms of the animal studies stress the inconsistent findings between species, low statistical power, and the use of high, environmentally unrealistic doses of TCE. Recently, several groups reported on possible mechanisms by which TCE may affect heart development in chick embryos (Drake et al., 2006a, b; Mishima et al., 2006) at more relevant doses in the ranges of 8 ppb to 80 ppm. Using the chick model, we previously showed that the epithelial-mesenchymal transition, a process leading to formation of cardiac valve precursors in the heart, was inhibited ex vivo in the 50–250 ppm range (Boyer et al., 2000). Because TCE cardiac teratogenesis remains a highly controversial issue, studies elucidating the molecular and cellular mechanisms of its action may help to clarify some aspects of the controversy (e.g., effects of low versus high doses of TCE, cell-specific effects versus whole organism). Ou et al. (2003) used cultured bovine coronary endothelial cells to demonstrate that TCE disrupted the activity of the endothelial nitric oxide synthase protein and endothelial cell function, suggesting a possible mechanism by which TCE may alter specific processes during heart development and contribute to heart malformations.

Similarly, previous molecular studies of the effects of TCE on rat embryonic hearts and a rat myocardial cell line in our laboratory showed that several molecules relevant to Ca\(^{2+}\) metabolism are perturbed. Collier et al. (2003) showed Serca2a mRNA expression to be reduced in rat embryo heart tissues collected at day 12 after daily maternal exposure to 100 ppm in drinking water. More recently, Ryr and CamK were identified in a microarray analysis of genes altered by exposure of a P19 mouse stem cell line to 1 ppm TCE in vitro (Selmin et al., 2008).

In cardiomyocytes, Ca\(^{2+}\) is a fundamental regulator of contractility and relaxation (Fig. 1). A heart beat contraction is...
activated the IP3 pathway. Local production of IP3 results in release of Ca2+ which induces PLB to release its inhibition from Serca2a. Serca2a then pumps Ca2+ from the cytosol into the SR for the next cardiac cycle. Ca2+ signaling can also be initiated by VP through the G-protein coupled AVP receptor to directly cause release of Ca2+. Although the Ca2+ entry via the sarcoplasmic reticulum (SR) storage and thus increasing intracellular Ca2+ concentration ([Ca2+]i) around the myofibrils. The relaxation phase occurs when CamKII phosphorylates phospholamban (PLB), which, in this form, relieves its inhibition of the SR Ca2+-ATPase (Serca2a) and allows Serca2a to quickly pump Ca2+ out of the cytosol and into the SR. This prepares Ca2+ stores (i.e., the SR) for the next contraction. Molecular defects in these Ca2+ channels or pumps can cause cardiac pathophysiology due to their role in excitation-contraction (Prasad et al., 2004). Reduced expression of Serca2a protein is associated with impaired heart function and malformations (Ki et al., 2000; Lalli et al., 2001; Periasamy et al., 1999; Ver Heyen et al., 2001). Furthermore, targeted overexpression of the Serca2a gene in transgenic mouse hearts has been shown to alter cardiac contractility by increasing SR Ca2+ transport (Baker et al., 1998). Ca2+ signaling can be directly activated in primary cultured rat cardiomyocytes through application of extracellular vesopressin (VP) (Chandrashekhar et al., 2003). Although application of VP results in an IP3-dependent release of Ca2+ from intracellular stores, many of the processes that contribute to [Ca2+]i regulation are shared with L-type channel initiated contractions.

In this paper, we present evidence that expression of the Serca2a and Ryr2 transcripts is repressed by TCE in a rat cardiomyocyte cell line (H9c2) consistent with our previous observations in vivo (Collier et al., 2003). We also show that the Ca2+ response to a natural agonist is altered by TCE, in both a dose- and time-dependent manner. These results suggest the toxic effects of TCE during embryonic cardiac differentiation might lead to development of congenital malformations in vivo via a dysregulated Ca2+ signaling pathway. The early requirement for cardiac function in normal development and the sensitivity of this process to perturbation of Ca2+ homeostasis may provide a working hypothesis to explore the apparent specificity of TCE as a cardiac teratogen in the developing rodent embryo.

MATERIALS AND METHODS

Materials

TCE was purchased from Aldrich (Milwaukee, WI). All other reagents were obtained from Sigma Chemical Co. (St Louis, MO), unless otherwise stated.

H9c2: Rat cardiac myocyte cell line. The rat cardiomyocyte cell line H9c2 was obtained from ATCC (CRL-1446). Cells were grown to 90% confluence in T-75 tissue culture flasks, in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 3.3 g/l NaHCO3 at 10% CO2. TCE solutions were prepared fresh daily for each treatment by diluting the original TCE reagent in DMEM–10% FBS media to obtain a 1000 ppm stock solution. Air space within the original TCE reagent bottle and stock solution was flushed with nitrogen gas to reduce chemical breakdown. Medium and fresh prepared TCE were replaced every 24 h. Control cultures (0 ppb) were incubated in a separate incubator to avoid any opportunity for volatile transfer.

For Real-Time PCR analysis, culture flasks were separated into treatment groups distinguished by TCE concentration: control, 10 ppb, 100 ppb, 1 ppm, 10 ppm; and time of TCE exposure: 12 and 48 h. Total RNA was extracted from H9c2 cells using Trizol LE reagent (Invitrogen, Carlsbad, CA) and purified using RNasy Mini Kits (Qiagen, Valencia, CA). Equal aliquots of total RNA were transcribed into cDNA using IScript supermix kit (Bio-Rad Laboratories, Hercules, CA), according to manufacturer protocols.

For calcium imaging analysis, cells were transferred to 15-mm glass coverslips (Carolina Biologicals, NC). Coverslips were placed in 25 mm wells in 12-well cell culture dishes (#3512, Corning Incorporated, Lowell, MA) and wells were filled with 1 ml of media. Each coverslip was separated into treatment groups: control, 10 ppb, 100 ppb, 1 ppm, 10 ppm. Within each group, cells were exposed to TCE for two different time periods: 18 and 52 h. Total RNA was extracted from H9c2 cells using Trizol LE reagent (Invitrogen, Carlsbad, CA) and purified using RNasy Mini Kits (Qiagen, Valencia, CA). Equal aliquots of total RNA were transcribed into cDNA using IScript supermix kit (Bio-Rad Laboratories, Hercules, CA), according to manufacturer protocols.

For Real-Time PCR

cDNA concentrations from each treatment were measured by spectrophotometry and equal amounts were used as templates for amplification. The Applied Biosystems Primer Express program was used to design primers and probes in regions specific to Serca2a (GenBank Accession Number gi 34872410), Ryr2 (GenBank Accession Number AF363960), and β-actin (GenBank Accession Number NM031144). Blast analyses of primers were performed against the rat genome to confirm their specificity. Efficiency of PCR amplification for each set of primers was determined using an external standard curve, generated using recombinant pCR2.1 plasmids containing amplicon fragments of the Serca2a, Ryr2 or β-actin genes. Real-time reactions were performed using QuantiTect Multiplex PCR kit (Qiagen, Valencia, CA) and run at a final volume of 25 μl. Each master mix consisted of the following: 12.5 μl QuantiTect Multiplex master mix, 2.5 μl of Serca2a probe (2μM),
1.0 μl reverse Serca2a primer (10μM), 1.0 μl forward Serca2a primer (10μM), 1.25 β-actin probe (2μM), 0.5 μl forward β-actin primer (10μM), 0.5 μl of reverse β-actin primer (10μM), 2.0 μl of template cDNA, and 3.75 μl of nuclease-free dH2O. Ryr2 PCR was conducted by SYBR Green analysis using ITAQ SYBR Green supermix with ROX (Bio-Rad Laboratories, Hercules, CA). Reactions were carried out using an ABI 7300 Real-Time PCR system and software (Applied Biosystems, Foster City, CA). Analysis was performed in triplicate, using cDNA samples from three independent experiments to account for biological variances. Concentration of each experimental sample was determined using the linear regression obtained from the serial dilution of the plasmid DNA standard. All samples were then normalized against β-actin. Expression levels in each treatment group were compared to untreated samples (control) and expressed in fold change.

**Primers for Real-Time PCR**

The primers used for PCR measurement were the following: Serca2a (Acc. Number gi34872419) 96889F: 5'-TTCACATCCTACACTAAAC3'; 98678R: 5'-TGGAATGTGGTGAACACG3'; Serca2a probe: 6FAM-ACTGGAGTAACCGCTTCTAAACATTGCAAG-TAMRA; Ryr2 (Acc. Number AF363960) F49: 5'-ACCGACGAGGAGGTTGGTG; R133: 5'CCACCCTGGATCTCCCAAGG; β-Actin (Acc. Number NM031144) 441F: 5'-CGCATGATTTTGGAACCTCACA3'; 527R: 5'-GGTGACGACCGAGGCATACA-3'; β-Actin probe 471 T: VIC-AGGAGTACGTAGCCAT-MGBNFQ.

**Real-Time PCR Standard—Plasmid Construct Design**

Fragment of the β-actin, Serca2a, and Ryr2 transcripts flanking their specific primers (see above) were isolated from H9c2 control cDNA. PCR products were separated on 1% tri-c-EDTA-agarose gel to confirm the correct insert size, and sent to The University of Arizona Sequencing Core (DNA Sequencing Service, Tucson, AZ) for sequence verification. PCR products were then sub cloned in pCR2.1 plasmid with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Plasmid concentration (number copy/μl) was calculated using Real-time standard curve linear regression and plasmid spectrophotometer readings.

**Cell Imaging**

H9c2 cells on glass coverslips were washed with a modified Hank's Balanced Saline Solution (HBSS: 1.3mM CaCl2, 5.0mM KCl, 0.3mM KH2PO4, 0.5mM MgCl2, 0.4mM MgSO4, 137.9mM NaCl, 0.3mM Na2PO4 and 1% glucose additionally buffered with 25mM 4-(2)-Hydroxyethyl piperazine-1-ethanesulfonic acid, pH 7.4) and loaded for 45 min in 5μM fura-2 AM (CalBiochem, La Jolla, CA). Cells were removed from fura2-AM loading solution and placed back into HBSS for at least 45 min before Ca2+ imaging. Fura 2 fluorescence was observed on an Olympus IX70 microscope with a 40× oil objective after alternating excitation at 340 and 380 nm by a 75-W Xenon lamp linked to a Delta Ram V illuminator (PTI, Inc., NJ) and a gel optic line. Images of emitted fluorescence above 505 nm were recorded by a ICCD camera (PTI, Inc., NJ) and simultaneously displayed on a 21” vivitron color monitor. The imaging system was under software control (ImageMaster, PTI) and collected 340/380 ratios approximately every 0.6 s. Intracellular Ca2+ concentration ([Ca2+]i) was calculated by ratiometric analysis of fura-2 fluorescence using equations originally published in (Grynkiewicz et al., 1985). A typical field of view contained between 8 and 17 cells (13.88 ± 2.04) for the 18-h treatment groups and between 10.17 ± 2.04 for the 52-h experiments. For each TCE treatment, five different fields were analyzed (except 18 h 10 ppm TCE, n = 4) in two separate treatment days, and represent data from at least 60 cells. In each field, only those cells that increased [Ca2+], > 100nM within 10 s were considered “responsive”. On average, 85-90% of cells in each field were considered responsive to VP and analyzed.

**Statistical Analysis**

All calcium imaging data were compared with GraphPad Software (San Diego, CA) using ANOVA with Tukey’s multiple comparison post-test. Real-time PCR data were normalized by fold expression as described in “Material and Methods,” and analyzed using Statview (SAS Institute, Cary, NC) statistical analysis software for ANOVA, multiple comparisons were by Fisher’s protected least significant different test. A value of p < 0.05 was used to establish significant differences between samples. Figures are graphed ± SEM.

**RESULTS**

**TCE Effects on Serca2a and Ryr2 Expression in Rat Cardiomyocytes**

Real-time PCR was used to determine whether Serca2a and Ryr2 mRNA expression was perturbed by varied doses and times of TCE exposure. H9c2 cells were exposed to control (0 ppb), 10 ppb, 100 ppb, 1 ppm, and 10 ppm TCE for 12 or 48 h. The wide range of TCE concentration was selected because other studies had shown a biphasic regulation of gene expression when low (ppb) versus high (ppm) doses of TCE were used. At 12 h after TCE exposure (Fig. 2A), cells displayed significantly reduced Serca2a transcripts at 10 ppb, 100 ppb, and 1 ppm by 22, 29, and 26%, respectively, compared to the controls. At 48 h (Fig. 2B), we observed overall a more pronounced inhibitory effect of TCE on Serca2a transcript levels. The most significant changes, compared to the control, were observed again, at 10 ppb, 100 ppb, and 1 ppm by 43, 41, and 47%, respectively. At the highest dose tested, 10 ppm TCE, lower average transcript levels were observed but were not significant due to the variability of the cellular response. TCE had similar effects on Ryr2 mRNA at 12 h (Fig. 2C), causing a reduced expression level at 10 ppb, 100 ppb, and 1 ppm TCE, but no significant change at 10 ppm TCE. At 48 h (Fig. 2D) we observed significant reduced expression at 10 ppb and 1 ppm, whereas 100 ppb and 10 ppm showed variable levels of expression, but not different from controls.

**TCE Effects on Ca2+ Signaling in Rat Cardiomyocytes**

To evaluate whether the changes in Serca2a and Ryr2 transcript level produced by TCE had a measurable effect on Ca2+ signaling in cardiomyocytes, H9c2 cells exposed to varying concentrations of TCE were loaded with Fura2 dye, washed with 1nM VP and [Ca2+]i changes over time were determined by ratio-imaging (Figs. 3–5). A typical H9c2 [Ca2+]i response to VP is shown in Figure 3. Variations in response to TCE included several measurable events: rate of increase in [Ca2+]i, in response to VP addition (Fig. 3A); the peak [Ca2+]i response to VP (Fig. 3B); the initial rate of recovery from the Ca2+ peak (Fig. 3C); and the [Ca2+]i flux in response to VP (Fig. 3D).

To approximate the intervals required for translation of transcripts measured in the previous experiments, we measured calcium fluxes in cells after 18 and 52 h of TCE treatment. After 18 h of TCE exposure, cellular response time to 1nM VP were significantly delayed in cultures treated with 10 ppb (~14 ± 2.05 s) or 1 ppm TCE (~11 ± 2.04 s) (Fig. 4A). In contrast, the intervening 100 ppb and the higher 10 ppm TCE exposure cellular response times were not significantly different from the
control. Peak [Ca] were significantly reduced in all four TCE doses, with 10 ppb and 1 ppm showing the greatest reduction (Fig. 4B). In control experiments, peak [Ca$^{2+}$]$_i$ averaged 400nM, where 18 h after TCE exposure significantly reduced this peak to 169nM for 10 ppb, 313nM for 100 ppb, 240nM for 1 ppm, and 311nM for 10 ppm.

The total change in [Ca$^{2+}$]$_i$ after 1nM VP can be approximated by evaluating the area under the Ca$^{2+}$ trace of a single experiment (e.g., C in Fig. 3). Total [Ca$^{2+}$]$_i$ change (area under the curve, Fig. 4C) was significantly reduced with all TCE treatments after 1nM VP. After exposure to our lowest dose, 10 ppb TCE, the largest decrease of 41% was observed in total [Ca$^{2+}$]$_i$. A loss of total [Ca$^{2+}$]$_i$ was also observed for 100 ppb, 1 ppm, and 10 ppm TCE by 17, 22, and 16%, respectively.

From the information recorded in each [Ca$^{2+}$]$_i$ peak, we calculated the slope to represent the changes of expulsion and intake of Ca$^{2+}$ from the SR. The “Rise to peak” slope again showed a bimodal effect from increasing doses of TCE with reductions at 10 ppb (82% decrease) and 1 ppm (64% decrease) (Fig. 4D). We found a reduced “Calcium Recovery” following TCE exposure, although these changes reached significance in only the 10 ppb (71% decrease) and 1 ppm (62% decrease) treated samples (Fig. 4E). Thus, although “peak calcium” and “area under the curve” were perturbed at all doses tested, measures of “time to peak,” “rise to peak,” and “calcium recovery” were altered only with 10 ppb and 1 ppm TCE.

To evaluate further damage or recovery after repeated exposure to TCE (fresh TCE was added every 24 h), we performed Ca$^{2+}$ evaluations in cultures treated with TCE after 52 h (Fig. 5). Under these conditions, no delays in the peak [Ca$^{2+}$]$_i$ response time were observed at the lower TCE doses (10 ppb and 100 ppb), however, as TCE was raised to 10 ppm, a significant reduction in response time, 7.3 s, when compared to control was observed (Fig. 5A). In contrast to the reduction seen at 18 h, TCE repeated exposures were found to increase both the peak change in [Ca$^{2+}$]$_i$ (Fig. 5B) and the total change in [Ca$^{2+}$]$_i$ (Fig. 5C). In control experiments, peak [Ca$^{2+}$]$_i$ averaged 315nM and TCE raised this peak to 362nM with an initial dose of 100 ppb and 363nM with 10 ppm. Total [Ca$^{2+}$]$_i$ change graph shows a trend of increased [Ca$^{2+}$]$_i$ with significance at 100 ppb, 1 ppm, and 10 ppm TCE by 31, 24, and 16%, respectively.
46%, respectively. The “rise to peak” (Fig. 5D) and “calcium recovery” (Fig. 5E) slopes showed the bimodal response seen at 18 h, but after 52-h treatments displayed considerable variability and the differences between the TCE exposed cells and controls were not significant.

**DISCUSSION**

**Serca2a and Ryr2 Expression**

During development, the cardiac level of Serca2a mRNA is low and gradually increases, reaching its maximum in the adult stages (Moorman et al., 2000). As Serca2a transcript is relatively rare in the early stages of differentiation, small changes in expression levels, such as those determined in this study, are likely to be physiologically significant. In fact, relatively small decreases in Serca2 levels observed in diseased hearts (Mercadier et al., 1990) can make considerable differences in contractile function (Pieske et al., 1999). Reduced mRNA levels of Serca2a were significantly reduced in failing hearts of rabbits (Armoundas et al., 2007) and Serca2 heterozygous null mice have physiological deficits (Periasamy et al., 1999).

Western blotting and biochemical analysis of heart tissue from heterozygous Serca2a-null mutant mice showed that Serca2 mRNA was reduced by 45%, whereas protein and maximal velocity of Ca$^{2+}$ uptake into the SR were reduced by 35% (Periasamy et al., 1999). In H9c2 cells, the level of Serca2a mRNA is normally low, consistent with the notion that these cells represent immature cardiomyocytes. These cells were chosen as they provide a model for rat cardiomyocytes, and because they have been extensively used for studies concerning cardiac defects, disease, and toxicant exposure. In order to evaluate long lasting effects of exposure to TCE, we chose to determine the mRNA expression levels of Serca2a and Ryr2 after 12 and 48 h from initial exposure to various doses of TCE. These times were chosen based on time course experiments showing that in H9c2 cells TCE effects on the Serca2a transcript were time-dependent and long lasting (Selmin et al., unpublished observations).

An analysis of the kinetics of exposure in culture showed that when TCE is added to the media, 80% of the initial dose is lost within the first hour, due to TCE’s volatile nature (Mishima et al., 2006). In the present study, therefore, the concentrations provided refer to initial TCE exposures, as decay in TCE is rapid enough that no measurable TCE concentration in the media can be determined accurately without compromising cell viability, especially when starting from as low doses as 10 ppb TCE.

The mRNA expression data show an overall inhibition by TCE on Serca2a and Ryr2 mRNA, both after 12 and 48 h. These results are consistent with previous reports indicating that reduced expression of Serca2a effects cardiac development.
and function (Haghighi et al., 2001; Hobai and O’Rourke, 2001). In particular, we observed that the lower concentrations of TCE used in the study (10 ppb–1 ppm) were effective in repressing gene expression after 48 h from initial exposure, whereas the highest dose (10 ppm) had no significant effect. The contrasting effects of low versus high doses are not unique to our findings. The same phenomenon was described by others comparing the effects of different doses of TCE on mesenchymal cell proliferation in avian embryonic hearts (Boyer et al., 2000; Drake et al., 2006a, b; Mishima et al., 2006). Possible explanations for these findings include activation of TCE metabolizing enzymes occurring at the higher TCE doses, and/or different levels of these enzymes in specific cell types and developmental stages (Drake et al., 2006a, b; Goldberg et al., 1992; Loeber et al., 1988). It is also important to note that other teratogens (e.g., ethanol) have shown similar dose-dependent behavior (He et al., 2007).

This observation that TCE does not produce a consistent dose-response curve contributes to the controversy regarding this solvent, but likely points to different affected substrates and multiple metabolites.

A reduced level of Serca2a and RyR2 expression at 12 h is consistent with the results obtained by calcium imaging, specifically with the slow “calcium recovery” and “rise to peak” showed by H9c2 cells after 18 h from TCE exposure. In cardiac myocytes, RyR2 proteins control the flux of calcium from the SR into the cytoplasm, where as calcium recovery inside the SR is controlled mainly by the activity of Serca2a.

Conditions in which the number of RyR2 and Serca2a complexes or their ability to function, are reduced, would likely produce the effects illustrated in Figures 4D and 4E. Therefore, it is tempting to speculate that Serca2a and RyR2 expression and function are both inhibited after 12–18 h from initial TCE exposure. On the other hand, after 52 h, calcium release in the cytoplasm (Fig. 5D) and its recovery (Fig. 5D) appear to be improved, suggesting that cells may activate mechanisms to counteract the persistent high calcium concentrations in the cytosol, including an increased expression of RyR2 and Serca2a, or that recovery has taken place as TCE has volatilized from the culture media.

**Ca**$^{2+}$ **Response in H9c2 Cells**

The data presented in Figure 4 indicate that H9c2 cells react slower and less efficiently to VP after 18 h from initial exposure to TCE. Although we cannot rule out that a component of this response might be due to an effect on VP receptors or activity, the slope of the response is consistent with a loss of RyR2 and the measured loss of RyR2 mRNA in the interval preceding calcium measurement. There were cells that were poorly responsive to VP in both control and treated cell populations and represented between 5 and 10% of all cells in each field analyzed (see “Materials and Methods”).

In cardiomyocytes, RyR2 is essential to the SR Ca$^{2+}$ release and amplitude of the peak [Ca$^{2+}$]i, therefore, our results indicate that TCE alters RyR2 function. The real-time PCR

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**FIG. 5.** H9c2 Ca$^{2+}$ Response to TCE after 52 h. H9c2 cardiac cells were cultured in the absence (0 ppb) or presence of 10 ppb–10 ppm TCE for 52 h. Cells were then stimulated with 1nM VP and evaluated for Ca$^{2+}$ parameters described in Figure 3. (A) through (E) are as described in Figure 4. *Denotes significant difference compared to 0 ppb as determined by ANOVA (p < 0.05).
data suggest that this is accompanied by a loss of transcripts and the data probably reflect a loss of protein combined with turnover of pre-existing molecules. These results argue that up to 18 h after exposure, TCE may significantly delay the heart beat cycle, at levels between 10 ppb and 1 ppm (Figs. 4A, D), where as 100 ppb and 10 ppm TCE produced minimal effect. Although it is conceivable that TCE directly inhibits function of the RyR2 channels and Serca2a pump, we think it more likely that these changes reflect a loss of the two molecules as well as others. In subtractive hybridization and microarray studies, we found TCE to affect transcriptional regulation of a number of other molecules (Collier et al., 2003; Selmin et al., 2005). In agreement with our findings, Fu et al. (2006) found that mouse ES cell-derived cardiomyocytes lacking RyR2 showed a marked decrease in Ca$^{2+}$ upstroke transients, twitch contractions, and prolonged time to peak. In other studies, it was reported that defective RyR2 channels can lead to irregular Ca$^{2+}$ handling and this may represent a possible causative agent for heart failure and arrhythmias (Marks et al., 2002; Marx et al., 2000). Consistently with our own findings, Buck et al. (1999) reported that myocytes exposed to the chlorinated hydrocarbon δ-hexachlorocyclohexane exhibited reduced contractility and ryanodine receptor function, due to disrupted calcium flux from the SR.

Conversely, after a longer time (52 h) and repeated TCE exposure (twice), cells were able to compensate or recover from the inefficiencies observed at 18 h. In fact, the peak [Ca$^{2+}$]i change and the total amount of Ca$^{2+}$ flux inside the cells is greater, indicating that other players must now be involved in the overall maintenance and contracting abilities in the heart cells.

Alterations in intracellular Ca$^{2+}$ homeostasis is thought to be a result of altered Ca$^{2+}$ handling proteins working together, that is, Serca2a and RyR2. In heart failure, Ca$^{2+}$ uptake in the SR is diminished by Serca2a protein loss (Arai et al., 1993; Hasenfuss et al., 1994; Lehnart et al., 1998), by functional upregulation of the Serca2a inhibitor, PLB (Reiken et al., 2003), or RyR2 gene downregulation (Go et al., 1995). Such an impact on the loss of SR function may be recovered by upregulation of NCX protein expression. This idea is also supported by Armoundas et al. (2007) who showed that failing hearts with functionless Serca2a rely on NCX to remove cytosolic calcium. In another study of failing hearts, protein levels of RyR, Serca2a, and PLB were significantly down-regulated, whereas NCX protein was significantly upregulated (Lehnart et al., 2004). Thus, the recovery seen at the later time point in this study is consistent with normal types of homeostatic responses.

In regards to cardiac function, elevated [Ca$i$], due to prolonged impaired Serca2a function could inhibit calcium clearance, leading to muscle fatigue, reduced contractility, and eventually to heart failure (Periasamy and Janssen, 2008). Thus, a compensatory response to reduced calcium handling could result in further negative effects for the heart function.

This outcome is consistent with our mRNA findings and with the observation that cells exposed to TCE for 52 h show a slightly higher baseline [Ca$i$] (10–14%) at increasing TCE concentrations when compared with controls (data not shown). Further, mitochondria could use a phosphate uptake mechanism to pump Ca$^{2+}$ into the matrix when the cytosol has high levels of Ca$^{2+}$ for an extended period of time. This has been reported to cause a calcium phosphate precipitate in the mitochondria matrix leading to calcification and damage to the mitochondria (Opie, 2004).

Dose-Response to TCE

One of the recurring issues in evaluating TCE as a cardiac teratogen has been the dose-response relationship (Dugard, 2000; Hardin et al., 2005). The NRC (2006) in its recent assessment of the developmental toxicity of TCE noted that studies in the rat model showed an unusually flat dose-response curve. The data presented here show a dose sensitivity that is lower than the majority of previous studies. In our own earlier studies we had noted an in vitro inhibition of epithelial-mesenchymal transition by cardiac valve precursors in the range 50–250 ppm of TCE (Boyer et al., 2000). This dose is consistent with a loss of valvular mesenchyme in vivo seen by Mishima et al. (2006) in the 10–80 ppm range. A loss of mesenchyme would be expected to produce valvular insufficiency and membranous septal defects as seen in exposed populations (Goldberg et al., 1990).

The present data are consistent with recent reports by Drake et al. (2006a, b) showing that in ovo exposure to doses between 8 and 800 ppb produced a proliferation of cardiac mesenchymal cells in the cardiac cushions and a reduced aortic flow in developing chick embryos. Increased cellular proliferation would likely be relevant to the etiology of the pulmonary stenosis noted in the Goldberg et al. (1990) study. Although attributed to a valvular stenosis, the reduction in aortic flow noted by Drake et al. (2006a) would also be consistent with a reduction in muscle contractility produced by a loss of calcium signaling as seen here. We note that reduced blood flow in the developing embryo is sufficient to produce morphological defects in cardiac structure (Groenendijk et al., 2004). Although we used an in vitro system, our data, if confirmed in vivo, are supportive of the idea that the toxic activities of TCE are due to the presence of multiple targets in cardiac tissue and perhaps differential effects of TCE metabolites. Thus the spectrum of cardiac defects noted in the study by Goldberg et al. (1990) may be indicative of varied exposures in the community as well as differential susceptibilities. Further studies using animal models and epidemiological data are warranted in order to prove or disprove this hypothesis.

In summary, our studies identify a major signal transduction pathway in cardiac muscle cells that is altered by exposure to low doses of TCE in a rat cardiac H9c2 myocyte cell culture model. As cardiac contraction during early embryonic
development is critical for normal heart morphology, the data suggest a basis for the particular sensitivity of the heart to TCE as a teratogen. Calcium fluxes are used as a second messenger mechanism in other cellular processes including the regulation of cardiac cushion formation (Runyan et al., 1990), but there may be less sensitivity to calcium perturbation in other developmental processes. Our ongoing studies will be examining the apparent regulation of transcription by low levels of TCE.

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