Original Article

Nrf2-Mediated Resistance to Oxidant-Induced Redox Disruption in Embryos

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Events that control developmental changes occur during specific windows of gestation and if disrupted, can lead to dysmorphogenesis or embryolethality. One largely understudied aspect of developmental control is redox regulation, where the untimely disruption of intracellular redox potentials (E_h) may alter development, suggesting that tight control of developmental-stage–specific redox states is necessary to support normal development. In this study, mouse gestational day 8.5 embryos in whole embryo culture were treated with 10 μM dithiole-3-thione (D3T), an inducer of nuclear factor (erythroid-derived 2)-like 2 (Nrf2). After 14 hr, D3T-treated and -untreated conceptuses were challenged with 200 μM hydrogen peroxide (H_2O_2) to induce oxidant-induced change to intracellular E_hs. Redox potentials of glutathione (GSH), thioredoxin-1 (Trx1), and mitochondrial thioredoxin-2 (Trx2) were then measured over a 2-hr rebounding period following H_2O_2 treatment. D3T treatment increased embryonic expression of known Nrf2-regulated genes, including those responsible for redox regulation of major intracellular redox couples. Exposure to H_2O_2 without prior D3T treatment produced significant oxidation of GSH, Trx1, and Trx2, based on E_h values, where GSH and Trx2 E_h recovered, reaching to pre-H_2O_2 E_h ranges, but Trx1 E_h remained oxidized. Following H_2O_2 addition in culture to embryos that received D3T pretreatments, GSH, Trx1, and Trx2 were insulated from significant oxidation. These data show that Nrf2 activation may serve as a means to protect the embryo from chemically induced oxidative stress through the preservation of intracellular redox states during development, allowing normal morphogenesis to ensue. *Birth Defects Res (Part B)* 95:213–218, 2012. © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

Recent work has highlighted the redox control of many different developmental processes. Cellular proliferation, differentiation, and apoptosis are tightly controlled by intracellular redox potentials (E_h), and chemically induced perturbation of E_h causes changes in these endpoints (Ranganna et al., 2007; Yan et al., 2009; Anathy et al., 2011). Most published work on redox regulation has primarily focused on events related to disease pathologies and has not directly addressed them during development. Since development is a combination of several molecular events that occur in a temporally coordinated manner, it is proposed that disruption of one or more of these events could, in turn, lead to a developmental deficit or birth defect (Hansen, 2006).

There are many teratogens that induce oxidative stress en route to producing adverse birth outcomes. The prototype teratogen thalidomide, for example, produces little cell death in embryos at teratogenic concentrations but still alters the developmental programing leading to limb dysmorphogenesis. In the past 15 years, many studies have shown that thalidomide effects have been shown to be caused primarily by the generation of reactive oxygen species (ROS) and disruption of redox signaling (Sauer et al., 2000; Hansen et al., 2002), suggesting that a primary oxidative mechanism by which some teratogens exert their effect is through the oxidative dysregulation of signaling.

In addition, many studies have shown the beneficial effects of antioxidant supplementation against teratogen-induced outcomes. The teratogenic effects of some of the most relevant teratogens to humans, such as ethanol, methyl mercury, and thalidomide, are all diminished through treatments with various antioxidants (Ornaghi et al., 1993; Parman et al., 1999; Parnell et al., 2010), suggesting that the maintenance of normal E_h values are critical for normal development.

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Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that activates the antioxidant response in cells and upregulates many genes associated with the regulation of intracellular redox states and ROS detoxification (Kwak et al., 2002; Thimmulappa et al., 2002). The purpose of the present study was to determine if Nrf2 activation in the embryo protects against subsequent oxidant-induced disruption of intracellular redox couples. Since certain redox couples regulate specific aspects of important signaling pathways, we have focused this study on the glutathione (GSH) and thioredoxin (both thioredoxin-1 [Trx1] and thioredoxin-2 [Trx2]) systems. Here, we demonstrate the effectiveness of Nrf2-mediated protection from oxidant-induced redox dysregulation in organogenesis-staged mouse embryos, suggesting a means by which teratogenesis may be mitigated.

METHODS

Animals and Whole Embryo Culture

Time-mated primagravida CD-1 (ICR) mice (ages 8–12 weeks) were purchased from Taconic (Hudson, NY) and were shipped directly to Emory University. Gestational day (GD) was assigned upon evidence of mating, with GD 0 beginning the morning following copulation and the discovery of a vaginal plug. Animals were cared for by the Division of Animal Resources at Emory University under a 12 hr/12 hr light/dark cycle in a temperature/humidity controlled facility and were given unlimited access to food and water until needed.

Whole embryo culture (WEC) was performed as fully described elsewhere (Sadler, 1979). On GD 8.0, mice were euthanized, and uteri were removed. In Tyrode's buffer (Sigma, St. Louis, MO), each uterus was dissected to remove each implantation site. Maternal tissues and Reichert's membranes were carefully removed to leave a conceptus with an intact visceral yolk sac. At this stage, embryos had three to six pairs of somites. Conceptuses were placed in culture medium consisting of heat-inactivated rat serum and Tyrode's buffer (3:1) supplemented with antibiotics (penicillin and streptomycin). Up to five conceptuses were placed into 5 ml of media. Bottles were fitted into a constant gassing rotating culture device (BTC Engineering, Cambridge, UK) heated to 37°C and gassed with 5% O_2, 5% CO_2, and 90% N_2 for 20–22 hr. At GD 8.5 (12 hr after the initiation of WEC), some conceptuses were treated with 10 μM dithiole-3-thione (D3T), a known inducer of Nrf2 and the antioxidant response, overnight (~12 hr). The next morning (GD 9) culture bottle media and headspace were saturated with 20% O_2, 5% CO_2, and 75% N_2. Embryos were treated with 200 μM H_2O_2 by direct addition to the culture medium 2 hr after the gas change. Sample embryos were first collected before the 200 μM hydrogen peroxide (H_2O_2) treatment (0 min), and at 5-, 15-, 30-, 60-, and 120-min intervals thereafter for subsequent analyses. Data presented are a result of four independently performed experiments. The values for each time point represent the average from all four independently performed experiments where two to four embryos were collected and individually analyzed for each redox couple (each data point represents 8–15 embryos). Embryonic protein values after D3T treatments were not significantly different from untreated embryos (data not shown).

Quantitative Real-Time PCR Analysis of Nrf2-Regulated Genes

Embryonic RNA was collected with the RNAeasy kit (Qiagen, Valencia, CA). Complementary DNA was synthesized from collected RNA with the Quantitect DNA synthesis kit (Qiagen) and prepared for real-time fluorescence PCR using SYBR Green detection master mix (SABiosciences, Fredrick, MD) per the manufacturer's instructions in a Opticon 2 real-time PCR cycler (BioRad, Hercules, CA). All primers were purchased from SABiosciences specifically for real-time PCR analyses. Genes that were measured included NADPH quinine:oxidoreductase-1 (NQO1), heme oxygenase-1 (HO1), glutamylcysteine ligase, catalytic subunit (Gclc), glutamylcysteine ligase, modifier subunit (Gclm), glutathione-S-transferase mu 2 (GST mu2), peroxiredoxin-1 (Prx1), thioredoxin reductase-1 (TR1), Trx1, peroxiredoxin-3 (Prx3), thioredoxin reductase-2 (TR2), and Trx2. β-Actin was measured as a housekeeping gene for normalization purposes. Samples were analyzed by the ΔΔCt method and are represented as a fold-change from untreated embryos of the same gestational age.

Glutathione Measurements

GSH and glutathione disulfide (GSSG) were measured by high-performance liquid chromatography with fluorescence detection and are expressed as molar concentrations based on cell volume (Kirlin et al., 1999). Concentrations were used to calculate E_h using the Nernst equation as previously outlined elsewhere (Jones, 2002; Jones et al., 2002).

Thioredoxin Measurements

Thioredoxin E_h were determined using a redox immunoblot technique that has been previously described elsewhere (Halvey et al., 2005; Hansen et al., 2006). Resolution of oxidized and reduced Trx was achieved following derivatization with 4-acetoamido-4-maleimidylstilbene-2,2-disulphonic acid (AMS, Invitrogen, Carlsbad, CA) and was separated based on protein size on a nonreducing sodium dodecyl sulfate (SDS) gel. Embryos were sonicated briefly, after which proteins were precipitated in ice-cold trichloroacetic acid (10%) for 30 min at 4°C then centrifuged. Pellets were washed with 100% acetone and centrifuged. Pellets were then dissolved in lysis/derivatization buffer [20 mM Tris/HCl, pH 8, 15 mM AMS with 1% SDS] and incubated at room temperature for 3 hr in the dark. Oxidized and reduced Trx were separated on an SDS/15% polyacrylamide gel in the presence of nonreducing loading buffer. Trx1 and Trx2 detection was performed on separate gels. Immunoblotting was performed onto nitrocellulose and detection utilized a goat anti–human Trx1 primary antibody (American Diagnostica, Greenwich, CT) and a rabbit anti-Trx2 as the primary antibody. Secondary antibodies were the AlexaFluor 680 nm donkey anti-goat and goat anti-rabbit (Invitrogen) for Trx1 and Trx2, respectively. Membranes were scanned with and Odyssey infrared scanner (Li-Cor, Lincoln, NE). Densitometric values were collected with

the Odyssey scanning software and E_h were determined using band intensities and the Nernst equation, where E_o was −254 mV for Trx1 at pH 7.4 and −330 mV for Trx2 at pH 7.6.

Statistics

Statistical analysis was performed using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Differences in fold expression of genes, GSH E_h, Trx1 E_h, and Trx2 E_h were determined by one-way ANOVA fallowed by Tukey's post hoc test. Significant difference were established where $p < 0.05$.

RESULTS

Nrf2-regulated genes are upregulated by D3T in the embryo. Both NQO1 and HO1 were significantly increased with D3T treatments (Fig. 1). Activation of these genes are classic hallmarks of Nrf2 activation and induction ranged between two- and threefold increases as compared to untreated embryos of similar gestational age and stage. Glutathione related genes, namely, Gclc, Gclm, and GST mu2, also were highly upregulated, with observed expression increases of 2.5- to 3.5-fold as elicited by D3T treatments. Expression of Prx1, TR1, and Trx1, comprising the principal components of the Trx1 system and largely found in the cytoplasm, were also significantly elevated. Similarly, the primary redox components of the mitochondrial thioredoxin system, Prx3, TR2, and Trx2, were also significantly upregulated.

GSH Redox Status

Total GSH and GSSG concentrations were not directly affected by D3T treatment before the challenge with H_2O_2,

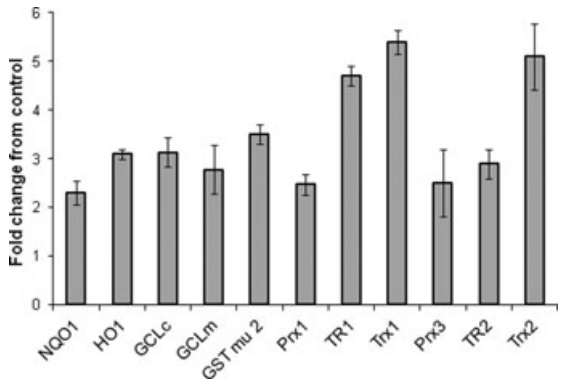

Fig. 1. Gene expression changes on known Nrf2 gene following a 14-hr treatment with 10 μM dithiole-3-thione (D3T) in gestational day (GD) 8.5 embryos. Changes in expression are denoted as fold changes from untreated embryos of the same gestational age. NQO1 (NADPH:quinone oxidoreductase-1), HO1 (heme oxygenase-1), Gclc (gamma glutamylcysteinyl ligase, catalytic subunit), Gclm (gamma glutamylcysteinyl ligase, modifier subunit), GST mu2 (glutathione-S-transferase mu 2), Prx1 (peroxiredoxin-1), TR1 (thioredoxin reductase-1), Trx1 (thioredoxin-1), Prx3 (peroxiredoxin-3), TR2 (thioredoxin reductase-2), and Trx2 (thioredoxin-2) were measured via real-time PCR as outlined in "Methods" section. Data represent 8–12 embryos per group from four separately performed experiments. Expression of these genes were significantly higher due to D3T treatments ($p < 0.05$).

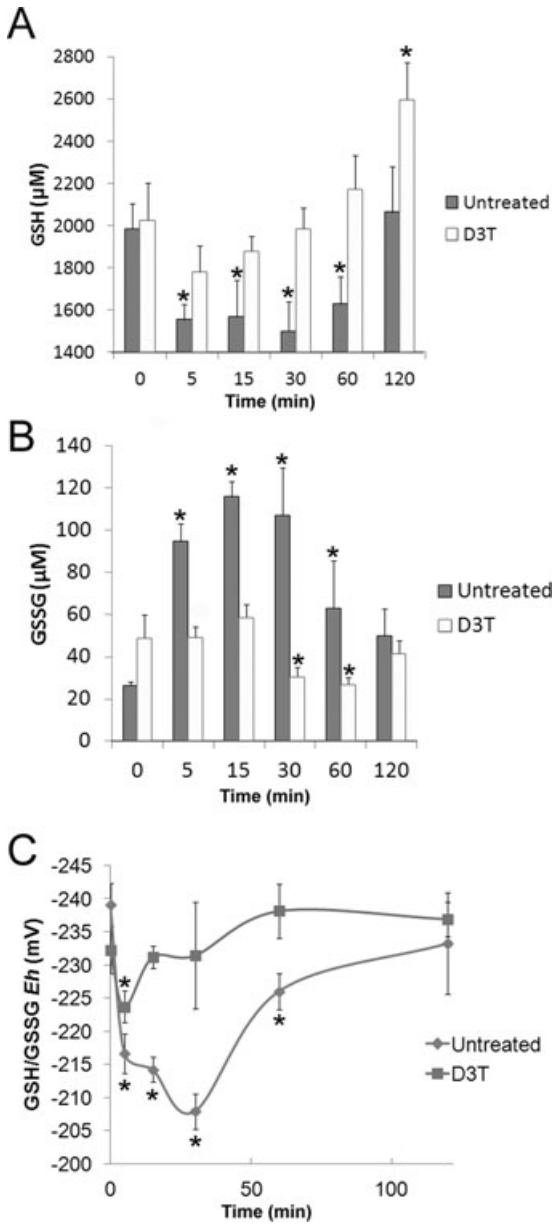

Fig. 2. Glutathione-related changes in embryos following H_2O_2 treatments with and without dithiole-3-thione (D3T) pretreatment over a 120-min period. (A) glutathione (GSH) and (B) glutathione disulfide (GSSG) concentrations are expressed as micromolar concentrations based on HPLC measurements. (C) GSH redox potentials expressed as millivolts (mV). Conceptuses pretreated with D3T showed less disruption of GSH redox status with H_2O_2 treatments compared to embryos not receiving the D3T pretreatment. Data represent 8–15 embryos per group from four separately performed experiments. Asterisks (*) denote a statistically significant difference ($p < 0.05$) from E_h at time 0, before H_2O_2 treatment.

although in D3T-treated embryos basal GSSG appeared to be slightly increased (Fig. 2A, B). This change did not result in significant differences in E_h. With H_2O_2 treatments, embryos that were not pretreated exhibited a decrease of GSH and a concomitant increase of GSSG, resulting in an oxidation of embryonic GSH E_h. This oxidation occurred quickly (5 min) and remained significantly oxidized for

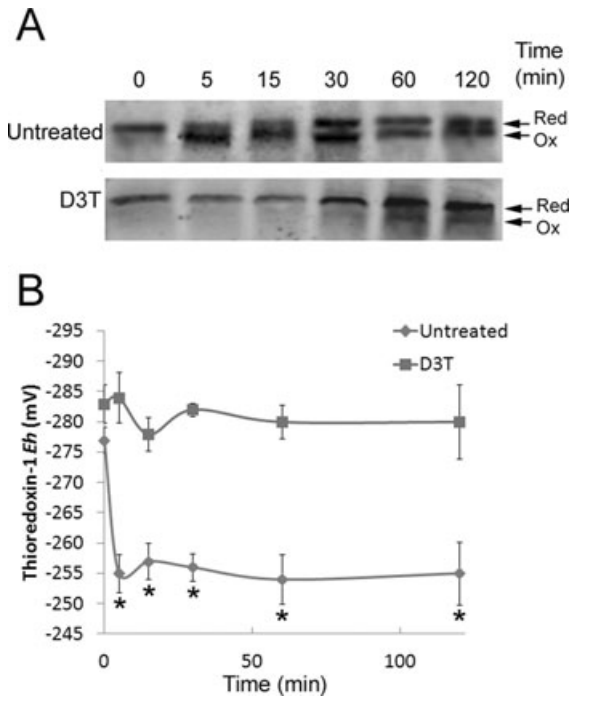

Fig. 3. Thioredoxin-1 redox changes in embryos following H_2O_2 treatments with and without dithiole-3-thione (D3T) pretreatment over a 120-min period. (A) Trx1 redox states were determined via redox Western techniques (see "Methods" section) to separate oxidized and reduced Trx1. (B) Using band densitometry, Trx1 redox potentials were determined and expressed as millivolts (mV). Trx1 was oxidized by H_2O_2 treatments that did not rebound over 120 min after commencement of oxidant treatment. Pretreatment of embryos with D3T treatments, prevented H_2O_2-induced oxidation of Trx1 even at early time points. Data represent 8–15 embryos per group from four separately performed experiments. Asterisks (*) denote a statistically significant difference ($p < 0.05$) from E_h at time 0, before H_2O_2 treatment.

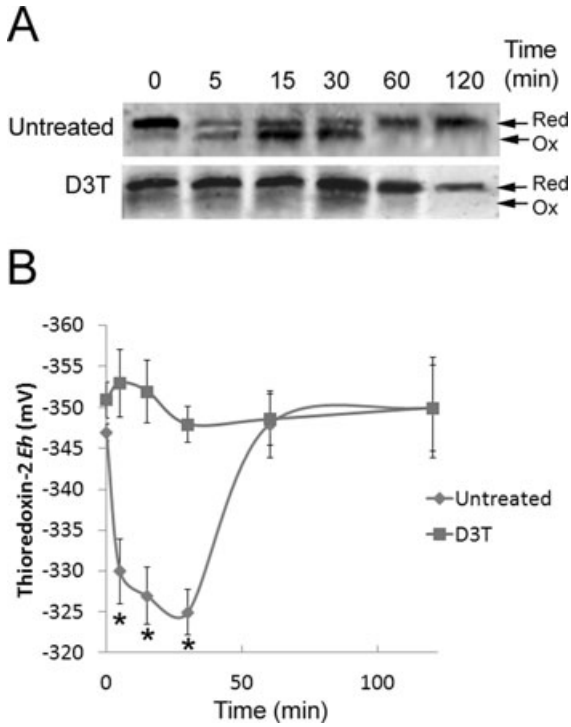

Fig. 4. Thioredoxin-2 redox changes in embryo following H_2O_2 treatments with and without dithiole-3-thione (D3T) pretreatment over a 120-min period. (A) Trx2 redox states were determined via redox Western techniques (see "Methods" section) to separate oxidized and reduced Trx2. (B) Using band densitometry, Trx2 redox potentials were determined and expressed as millivolts (mV). Unlike Trx1, Trx2 redox states did rebound after 30 min and was completely restored by 60 min in embryos that were not pretreated with D3T. With D3T pretreatments, embryos were not affected by H_2O_2 treatments and remained similar to baseline levels. Data represent 8–15 embryos per group from four separately performed experiments. Asterisks (*) denote a statistically significant difference ($p < 0.05$) from E_h at time 0, before H_2O_2 treatment.

60 min. At 120 min, the GSH E_h returned to baseline levels. With D3T pretreatments, H_2O_2 treatments caused a slight loss of GSH but produced no change in GSSG levels. While these were not significantly different from baseline levels, concentration changes were significant enough to produce an oxidation of GSH E_h, but were much less (+10 mV) compared to embryos not pretreated with D3T (+22 mV). However, by 15 min, GSH E_h was completely restored to normal values.

Thioredoxin-1 Redox Status

In control embryos, Trx1 E_h was severely oxidized by H_2O_2 treatment. At 5 min, Trx1 E_h was increased by +22 mV. Interestingly, Trx1 redox status did not rebound over the 120-min measurement period, but rather remained oxidized throughout. Conversely, with D3T pretreatment, embryos were fully resistant to H_2O_2 treatment, where Trx1 E_h were unchanged even at the earlier time points (Fig. 3).

Thioredoxin-2 Redox Status

Trx2 from embryos that did not receive the D3T pretreatments showed an oxidation after H_2O_2 treatments. Trx2 E_h remained significantly oxidized (up to +22 mV) for 30 min until the 60 min time point where it rebounded back to baseline E_h values. With D3T pretreatments, Trx2 was fully protected and no observable oxidation was detected (Fig. 4).

DISCUSSION

Changes in redox status are known to activate or deactivate transcription factors. Excessive oxidation of redox couples alters intracellular environments where proteins are susceptible to oxidative modification. Activator protein-1 (AP-1) contains a redox-sensitive cysteine residue that modulates its DNA binding (Abate et al., 1990). In embryos, depletion of GSH causes excessive AP-1 activation, increasing related gene expression while promoting concomitant dysmorphogenesis (Ozolins and Hales, 1997; Ozolins et al., 2002). Similarly, nuclear factor-kappa B (NF-κB) also contains cysteine residues in its DNA-binding domain that regulates its gene transactivation activity (Matthews et al., 1992). Interestingly, thalidomide embryotoxicity has been hypothesized to be mediated through ROS-induced dysregulation of NF-κB

(Hansen et al., 2002; Hansen and Harris, 2004). While both of these factors are important for normal development, they serve as examples of redox dysregulation of normal signaling that can occur in the embryo. Based on concepts developed in lower organisms and now applied to mammals, there are likely many other proteins that are susceptible to oxidative dysregulation in the embryo that may have equally detrimental outcomes during periods of oxidative stress (Allen and Balin, 1989; Hitchler and Domann, 2007).

Clearly, maintenance of normal, stage-specific redox states is important to allow normal signaling and development. However, the compartmental, subcellular nature of an oxidation may serve to promote specific responses. For example, Trx2 has been shown to be a critical factor for the regulation of mitochondrial function and membrane permeability transition (He et al., 2008). Other studies have shown that Trx2 prevents oxidant-induced apoptosis, and thus, periodic oxidation of Trx2 may serve as a means to promote apoptosis (Chen et al., 2002). Here, we demonstrate that the embryo re-regulated Trx2 redox status by 60 min, but it will require further study to determine if a 60 min Trx2 oxidation during this developmental stage has any detrimental downstream consequences.

Our data presented here show that both GSH and Trx2 rebound at approximately the same rate, suggesting that pathways regulated by these two redox couples are dysregulated for a short period of time (30–60 min) at this stage of development. Although GSH and Trx2 redox states are restored over time, the undesired changes to redox-sensitive signaling pathways are likely to occur on a more temporary basis. It is unclear, how these more transient redox shifts affect differentiation/development, but periods of sustained oxidation are likely to produce more profound effects in the embryo.

Our data show that Trx1 does not rebound but rather remains oxidized for at least 120 min after H_2O_2 treatment. Trx1 is a critical protein oxidoreductase that has been shown to participate in the regulation of critical cysteine residues in many proteins, including transcription factors, such as NF-κB, AP-1, and others (Mitomo et al., 1994; Jayaraman et al., 1997). Trx1's role occurs primarily through the redox control of redox factor-1 (Ref1). Ref1 is a nuclear protein that functions as a DNA repair enzyme AP endonuclease but also interacts with transcription factors to regulate their redox states and activity (Seki et al., 1991; Xanthoudakis and Curran, 1996; Nishi et al., 2002). After reducing its target, Ref1 becomes oxidized but can be recycled back to its reduced form through Trx1 (Hirota et al., 1997). Oxidized Trx1 is then reduced by TR1 to allow further oxidoreductase activity. In control, untreated embryos, we observed sustained oxidation in the embryo with peroxide treatment, suggesting that many Trx1-dependent processes, such as transcription factor activities, are likely to become altered during periods of oxidative stress where Trx1 redox states are pertrubed, promoting faulty signaling and possibly leading to embryonic malformation.

Nrf2 has been implicated in mediating the acute toxicities produced in many tissues, including those found in the embryo. Prior activation of Nrf2 by D3T or (-)-epigallocatechin-3-gallate (EGCG) was shown to protect embryos from ethanol-induced embryotoxicity in utero (Dong et al., 2008; Long et al., 2010). Moreover, treatment with another Nrf2 activator, *tert*-butylhydroperoxide (tBHQ), provided protection against ethanol in neural crest cells, a target of ethanol in embryos (Yan et al., 2010). Studies have shown that embryonic exposure to ethanol increases ROS generation, markers of oxidative damage, and a loss of reduced GSH (Henderson et al., 1999; Akella et al., 2000; Long et al., 2010), but these studies have failed to delineate the duration of oxidative stress, degree of redox imbalance and recovery profiles in the embryo. Here, we show that not only do different redox couples have very different recovery kinetics but much of the effects of oxidants may also be prevented through Nrf2 activation. Clearly, further work is needed to characterize and understand the role of Nrf2 during development and in adapting to environmental and chemical influences on the embryo proper.

Activation of Nrf2 through D3T treatment prevents prolonged periods of oxidation and in many cases prevents any oxidation following oxidant treatments in vitro. Nrf2-mediated protection from prolonged periods of redox imbalance, especially with Trx1, may serve as a means to inhibit oxidative stress-mediated teratogenesis. These studies suggest that many harmful substances that are ubiquitous in the environment (i.e., heavy metals, pesticides, etc.) that may promote teratogenesis through an ROS-mediated mechanism may be mitigated through proper diet and nutritional supplementation. Currently, these recommendations are poorly delineated, but should serve as a focus of research to protect the embryo from insult.

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