Overexpression of Nrf2 protects cerebral cortical neurons from ethanol-induced apoptotic death

Madhusudhanan Narasimhan, Lenin Mahimainathan, Mary Latha Rathinam, Amanjot Kaur Riar and George I. Henderson

MN, LM, MLR, AKR, GIH - Department of Pharmacology & Neuroscience,
MN, LM, GIH - South Plains Alcohol and Addiction Research Center, Texas Tech University Health Sciences Center, 3601 4th Street, STOP 6592, Lubbock, Texas, 79430 USA.
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**Address for Correspondence:**

George I Henderson; E-mail: george.henderson@ttuhsc.edu
(or)
Lenin Mahimainathan; E-mail: lenin.mahimainathan@ttuhsc.edu

Tel: 806-743-2425
Fax: 806-743-2744

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**Abbreviations:** Act D, actinomycin D; Ad WT Nrf2, adenovirus for wild type Nrf2; Ad DN Nrf2, adenovirus for dominant negative Nrf2; Ad GFP, adenovirus for green fluorescent protein, ARE, antioxidant response element; ETOH, ethanol; FAS, fetal alcohol syndrome; GSH, glutathione; HET, hydroethidine/dihydroethidium; MCB, monochlorobimane; PCNs, primary cortical neurons; ROS, reactive oxygen species; siNrf2, small interfering RNA against Nrf2; Scr, Scrambled siRNA.
Abstract

Ethanol (ETOH) can cause apoptotic death of neurons by depleting glutathione (GSH) with an associated increase in oxidative stress (Rathinam et al., 2006). The current study illustrates a means to overcome this ETOH-induced neurotoxicity by enhancing GSH through boosting Nrf2, a transcription factor which controls GSH homeostasis. ETOH treatment caused a significant increase in Nrf2 protein, transcript expression, the Nrf2-DNA binding activity and expression of its transcriptional target, NQO1 in PCNs. However, this increase in Nrf2 did not maintain GSH levels in response to ETOH and apoptotic death still occurred. To elucidate this phenomenon, we silenced Nrf2 in neurons and found that ETOH induced GSH depletion and the increase in superoxide levels were exacerbated. Further, Nrf2 knockdown resulted in significantly increased (P<0.05) caspase-3 activity and apoptosis. Adenovirus mediated overexpression of Nrf2 prevented ETOH induced depletion of GSH from medium and high GSH subpopulation and prevented ETOH-related apoptotic death. These studies illustrate the importance of Nrf2-dependent maintenance of GSH homeostasis in cerebral cortical neurons in the defense against oxidative stress and apoptotic death elicited by ETOH exposure.
Introduction

Exposure to ethanol during pregnancy, a debilitating public health problem is associated with adverse outcomes ranging from Fetal Alcohol Syndrome (FAS) to Alcohol Related Neurodevelopmental Disorder (ARND), which are collectively known as Fetal Alcohol Spectrum Disorder (FASD) (Sokol et al., 2003). A variety of animal and cell-based experimental FASD models employed in identifying the underlying mechanisms behind ethanol-induced oxidative stress-dependent developmental deficits have been comprehensively reviewed (Brocardo et al., 2011), yet such mechanisms and interventions remain elusive.

Previous studies from our laboratory and others in both in vivo and in vitro models have documented that the ethanol elicited developmental deficits affecting multiple regions of CNS are linked to reduced progenitor cell proliferation, disordered neuronal migration, disrupted neurotransmitter function, and enhanced neuron death (Gressens et al., 1992; He et al., 2005; Henderson et al., 1995; Jacobs and Miller, 2001; Ramachandran et al., 2001; Ramachandran et al., 2003). Though programmed cell death of neurons, is an obligatory process in the developing brain for its optimal development (Sastry and Rao, 2000), exposure to the pro-oxidant, ethanol, elicits an apoptotic response concomitant with a loss of redox homeostasis in neurons (Ramachandran et al., 2003).

In general, NF-E2-related factor 2 (NFE2L2/Nrf2), a basic leucine-zipper transcription factor tightly controls redox homeostasis and facilitates the adaptation of neurons to hostile oxidative environment (Lee et al., 2005). Under basal condition, Nrf2 is sequestered in the cytoplasm by a chaperone molecule, Keap1 which upon oxidant stimulation escapes from Keap-1, binding to ARE elements in nucleus and up-regulating the transcription of NQO1, phase II genes including those involved in GSH biosynthesis (Itoh et al., 1999; Kraft et al., 2004; Shih et al., 2003).
While, converging evidence from several fields demonstrated that Nrf2 deficient cells/animals is linked to the increased susceptibility to oxidant-mediated injury, the intact Nrf2 pathway is indispensable for mounting defense mechanisms against those toxic stressors (Kensler et al., 2007; Satoh et al., 2006; Shih et al., 2005a; Shih et al., 2005b). In a recent study, (Dong et al., 2008) have demonstrated a significant role for Nrf2 signaling in ETOH-induced teratogenesis in intact embryos. In addition, there are many studies elucidating a range of mechanisms involved in ethanol induced apoptosis in numerous cell types viz. cerebellar granule neurons (Bhave et al., 2000), neocortical neurons (Jacobs and Miller, 2001) and midbrain neurons (Crews et al., 1999). Evidence generated from our laboratory have illustrated that ethanol can accelerate apoptotic cell death of neurons in developing brain (Ramachandran et al., 2001; Ramachandran et al., 2003) and such an increase in neuron loss can be correlated to a reduction in progressive brain tissue volume shrinkage, cortical gray matter volume reduction, and ultimately to altered cortical functions (Pfefferbaum et al., 1998). Thus, connections between an ethanol-mediated damage to cells within the developing cerebral cortex and functional consequences of ethanol exposure are in place. Our current study is the first of its kind to focus specifically on the cerebral cortical region response of fetuses and neurons isolated from fetal cortices to ETOH with respect to a vital signaling event(s) involving Nrf2. It is further shown that adenovirus mediated overexpression of Nrf2 can protect fetal primary cortical neurons against ETOH-induced oxidative stress and apoptosis.

**Materials and Methods**

**Materials**

Minimum Essential media (MEM), Dulbecco Minimum Essential Medium (DMEM), Hank’s balanced salt solution (HBSS), fetal bovine serum (FBS), TRIzol were purchased from...
Invitrogen (Carlsbad, CA). Horse serum (HS), trypsin, DNase, antibiotics, poly-D-lysine, uridine, monochlorobimane, hydroethidine, actinomycin D, and tubulin monoclonal antibody were from Sigma (St. Louis, MO). Fisher-Costar cell culture inserts for co-culture were obtained from Fisher Scientific (Pittsburgh, PA). 4,6-diamidino-2-phenylindole (DAPI), Annexin-V FITC apoptosis detection kits were obtained from BD Biosciences(San Jose, CA). Nrf2 (c-20), GAPDH antibodies, NFE2 / Nrf2 / ARE consensus and mutant oligonucleotides were from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti microtubule-associated protein 2 (MAP2), glutathione detection kit was purchased from Chemicon (Temecula, CA). Anti-GFAP was obtained from Millipore (Billerica, MA). Smart Pool siRNA against Nrf2 and non-targeting siRNA pool was purchased from Dharmacon Inc., (Lafayette, CO). Quantitect reverse transcription kit for first stand synthesis was purchased from Qiagen Inc., (Valencia, CA). Cytosolic and Nuclear protein extraction kit, ECL chemiluminescence detection kit was purchased from Pierce Biotechnology Inc., (Rockford, IL). Caspase-Glo 3/7 assay kit was obtained from Promega Corporation (Madison, WI). siPORT amine was from Ambion Inc. (Austin, TX). TransAM ELISA kit for Nrf2 was purchased from Active Motif (Carlsbad, CA).

**Primary Cortical Neuron (PCN) Cultures**

PCNs were prepared from E16-E17 timed pregnant Sprague Dawley rats as described earlier (Dutton, 1990; Ramachandran et al., 2003). Briefly, embryos from amniotic sac were taken out and the cerebral cortex was carefully dissected out. Fetal cortex was mechanically dissociated in HBSS and the cells were suspended in MEM containing 10% FBS and 10% HS. The cells were seeded at a density of 1.75 x 10^6 cells/well in a 6 well plate previously coated with poly-D-lysine and the neurons were maintained in a humidified atmosphere of 95% air and 5% CO₂. After 1DIV, the cells were given “inhibitory” feeding with uridine (10mg/mL) containing MEM
supplemented with 10% HS to suppress the growth of astrocytes. Following 48 h, old media was replaced by fresh MEM containing 10% HS and subsequently, PCNs were subjected to treatment either on the 4th day of culture (for siRNA transfection and adenovirus infection experiments) or on the 5th day (for ETOH and Act D treatment). This is a well-established and documented primary neuronal culture system, which is essentially free of glia. Dual immunostaining with MAP2 (for neurons) and GFAP (for astrocytes) were performed and the isolation procedure adopted yielded ~95% enriched neuronal culture (Supplemental Figure 1).

**In Vivo Model**

A 2-day exposure regimen in an animal model of acute ethanol exposure was employed to mimic an alcohol binge in humans (Henderson et al., 1995). Sprague Dawley rats were intubated with ethanol (4 g/kg body wt, 25% v/v) at 12 h intervals on days 17 and 18 of gestation. Pair-fed control rats were weight matched to the ethanol-fed dams and was intubated with isocaloric dextrose. On day 19, 1 hour before sacrifice, a final dose was administered. Both control and ethanol-fed dams had full access to standard laboratory chow and water ad libitum, whereas pair-fed controls had full access to water but received the weight of chow consumed by the corresponding ethanol dam during the previous 24 h period. The gestational age of the pair-fed control and ethanol rats were staggered by a day, in order to ensure that animals from the pair-fed control received chow at the same stage of gestation as did the corresponding ethanol-treated dams. At the end of treatment, animals were sacrificed; Blood alcohol levels were determined using Analox AM1 analyzer. Fetal brain cortices were carefully isolated and stored in -80°C until use. All animal protocols were approved by Institutional Animal Care and Use Committee. Handling and treatment of animals were carried out according to the National Institutes of Health guidelines for the use and care of laboratory animals.
Ethanol treatment of PCNs

On 5 DIV, PCNs were treated with ETOH (4 mg/mL) for different time points (30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h) in an incubator saturated with ETOH to maintain media ETOH (monitored using Analox AM1 alcohol analyzer) (Rathinam et al., 2006). The *in vitro* experiments involving ETOH in the current study are short term (24 h) whereas alcohol abuse is usually prolonged. This is a clinically relevant, albeit high, concentration which is at or below that reported in mouse and rat models which expressed prominent brain apoptotic responses (Olney et al., 2002; Rathinam et al., 2006).

Actinomycin D treatment

PCNs on 5DIV were co-incubated with ETOH (4 mg/mL) and Act D (1 µg/mL). No adverse effects of Act D on PCNs were seen at this concentration and duration. Following treatment, cells were incubated for the above time periods, after which they were harvested, total RNA isolated and real time qRT-PCR for Nrf2 and GAPDH determinations were carried out.

Adenovirus Infection

PCNs seeded at a density of 1.75 x 10^6 cells/well in 6 well plates were infected at DIV4 with either Ad GFP or Ad WT Nrf2 or Ad DN Nrf2. The viruses were generously provided as a gift by Dr. Jeffrey Johnson (University of Wisconsin, Madison, USA). Virus infection was performed in complete media at a MOI of 200 for 1 h at room temperature. The plates were then returned to the incubator for additional 1 h and the media was replenished with fresh 10% MEM media. The infected plates were then returned to a 37° C humidified 5% CO2 incubator and the cells were harvested after 24 h and 48 h for western analysis of Nrf2 expression. All experiments involving 24 h ETOH exposure were carried out 24 h postinfection.
Small interfering RNA (siRNA) transfection

siRNA transfection experiments were performed in 4 DIV PCN in 6 well plates. 5 µL of siPORT amine diluted in Opti-MEM was gently mixed either with 100 nM of smartpool siRNA against Nrf2 or non-targeting siRNA pool. After complex formation according to manufacturer’s instructions (Ambion), the mixture was gently added to PCNs. Silencing was observed with western analysis for Nrf2 protein expression after 24 h and 48 h. For the experiments involving ETOH, 24 h post transfection of siRNA the cells were exposed to ETOH for additional 24 h and processed for various downstream applications such as western, FACS analysis for detection of MCB, HET, Annexin V FITC/PI, and the caspase glo assay.

RNA extraction and real time qRT-PCR analysis

Total RNA was isolated from PCN or cerebral cortex by the TRIzol reagent according to the manufacture recommendations (Invitrogen). 1.5 µg of total RNA were effectively removed of genomic DNA contamination and reverse transcribed by Quantitect reverse transcription kit (Qiagen). 1/10th of cDNAs were used in real time-RT PCR analysis of mRNA expression for Nrf2, GCLC and GAPDH. Taqman gene expression assays consisting of the primers and probes specific for rat Nrf2 (Rn00582417_g1) and rat GAPDH (Rn99999916_s1) were from Applied Biosystems (Bedford, MA). Real time PCR amplification was performed in 96-well optical plates in a final volume of 20 µL containing 10 µL of TaqMan Universal Mastermix (Applied biosystems), 20 pmol of respective primers and 1/10th of reverse transcribed RNA. For the experiment involving GCLC mRNA quantitation, SYBR green based real-time PCR assay (PA-012) was performed using real time primers for rat GCLC (PPR44067A) and rat GAPDH (PPR06557A) according to manufacturer’s instructions (SA biosciences, Frederick, MD). Real time PCR was conducted in an ABI Prism 7300 (Applied Biosystems). The expression of the
target gene (Nrf2, gamma-glutamylcysteine ligase (GCLC)) was determined relative to GAPDH as an internal control and the relative fold change in the mRNA expression was calculated using the $2^{-\Delta\Delta CT}$, where $\Delta CT=CT_{\text{target gene}}-CT_{\text{GAPDH}}$ and $\Delta\Delta CT=\Delta CT_{\text{treated condition}}-\Delta CT_{\text{untreated condition}}$.

**Reverse transcription-PCR analysis**

Total RNA was isolated from untreated and ETOH-treated PCNs and *in utero* brain cortices and cDNA was prepared as above. 1/10th of cDNA was used in PCR reaction using primers specific for rat NQO1 (For: 5’ – ACTCGGAGAACTTTCAGTACC – 3’; Rev: 5’ – TGGAGCAAAATGAGTGGT – 3’(Yan et al., 2008) and rat GAPDH (For: 5’ – AGACAGCCGCATCTTTCTTGT – 3’; Rev: 5’ – TACTCAGCACCAGCATCACC – 3’). After an initial denaturation at 95°C for 3 min, PCR specific for NQO1 was performed in a 25 µL-reaction volume for 35 cycles under the following conditions: 95°C for 30 s, 53°C for 30 s, 72°C for 60 s, and finally an extension at 72°C for 5 min. GAPDH cycling parameters were the same except for the annealing temperature which is 55°C for 30 s. Aliquots of the PCR product was run on 1% agarose gel and the products for NQO1 and GAPDH were visualized at 492 bp and 323 bp respectively by ethidium bromide staining using UVP gel documentation system. The images were photographed and quantified using Image J software.

**Western blot analysis**

PCNs or cerebral cortices were lysed in RIPA buffer at 4°C for 30 min and after sonication and centrifugation at 15,000 g for 15 min, the supernatant was used for immunoblot assay. Briefly, 25 µg of equal amounts of lysates from various treated groups were resolved by electrophoresis on 8% polyacrylamide gels, electrotransferred to a polyvinylidene difluoride filter (PVDF), and blocked with 5% non-fat dry milk. After incubation with primary antibodies against rat Nrf2, GAPDH, tubulin, lamin the membranes were then washed thrice in PBST and incubated with
HRP-conjugated anti-rabbit or mouse IgG or goat IgG for 1 h. The antigen-antibody complex was detected using ECL chemiluminescence kit. Nrf2 bands were quantified by scanning densitometry using Image J software and were normalized to the signal intensity of GAPDH.

**Double Immunofluorescence**

PCNs cultured on poly-D-lysine coated coverslips were treated with or without ETOH (4mg/ml; 30 min), washed with PBS, fixed in 2% paraformaldehyde, blocked as needed and followed by Nrf2 and MAP2 dual immunostaining. Nrf2 and MAP2 expression was detected using Cy3-conjugated donkey anti-rabbit antibodies and FITC-tagged donkey anti-mouse secondary antibodies respectively. DAPI was used to stain the nuclei to determine nuclear localization of Nrf2, if any. The cells were visualized and imaged using Olympus Fluoview 500.

**Electrophoretic mobility shift assay (EMSA)**

Nuclear proteins were extracted from PCN or cerebral cortex using a nuclear and cytoplasmic extraction kit (Pierce, IL). EMSA binding reactions were performed at room temperature in a final volume of 20 µL containing 10 µg of the nuclear extract from both control and varying time points of ETOH treated samples, 50000 cpm of ³²P-labeled Nrf2 oligonucleotide probe, 1 µg of poly dI-dC. Similarly, in a separate reaction, the corresponding mutated Nrf2 oligonucleotide was also incubated with the nuclear extracts. To determine the specificity of protein binding to the Nrf2/ARE sequence, competition assays were performed in the presence of a 100-fold excess of the unlabeled competitor oligo (Nrf2 consensus) along with nuclear extract for 10 minutes prior to the addition of radiolabeled probe. The supershift assays were performed by preincubating nuclear extracts with 2.5 µg Nrf2 antibody in ice for 15 min and the reaction was carried out at room temperature for 30 min after adding the radiolabeled probe. The DNA-protein complexes were resolved in a 5% non-denaturing polyacrylamide gel in 0.5x Tris-borate,
EDTA buffer and gels were dried, autoradiographed with intensifying screens at -80°C.

**ELISA based measurement of Nrf2 activity**

The TransAM™ Nrf2 Kit was used to assay the DNA-binding activity of Nrf2 in the nuclear extracts that were obtained from both the untreated and ETOH treated cells/brain cortices. Briefly, 5 µg of nuclear extracts from each samples in triplicates were incubated in a 96 well plate that is coated with oligonucleotide containing a consensus-binding site for Nrf2. For competitive binding experiments, which measure the specificity of the assay, 5 µg of nuclear extract from the ETOH treated cells were assayed in the presence of wild-type or mutated competitor oligonucleotides. After 1 h incubation the wells were washed and incubated with 100 µL of a 1:1000 dilution of rabbit polyclonal antibody against. Incubation with normal rabbit polyclonal IgG was also carried out separately to determine the specificity of Nrf2 antibody. The wells were then washed followed by incubation with 100 µL of a 1:1000 dilution of HRP-conjugated, anti-rabbit secondary antibody at room temperature. The wells were developed using 100 µL TMB substrate for 6 min and 3 min for cell lysates and tissue lysates respectively before adding 100 µL stop solution. Optical density was read at 450 nm with a reference wavelength of 650 nm using a plate reader from Biorad Laboratories (Hercules, CA).

**GSH measurement by flow cytometry**

Monochlorobimane a non-fluorescent reagent which reacts with GSH to form a highly fluorescent derivative, was used to detect free GSH in individual viable cells by flow cytometry (Maffi et al., 2008). At the end of treatment, neurons were incubated with 10 µM of MCB for 30 min in cell culture incubator, cells scraped, washed, and resuspended in cold PBS. Acquisition and analysis were performed on a FACS flow cytometer with excitation and emission settings of 360 nm and 460 nm, respectively.
GSH measurement by fluorimetric method

Intracellular levels of reduced GSH were assessed by glutathione detection kit (Chemicon). Nrf2 knockdown cells treated with or without ETOH for 24 h, were washed with 1.5 mL wash buffer followed by lysis and clarification using centrifugation. 90 µL of lysate were mixed with 10 µL of MCB and incubated in a black 96 well plate at room temperature in a light protected environment. Fluorescence was read in a fluorometer using a 380/460 filter set and the results were expressed as RFU.

Measurement of cellular superoxide by flow cytometry

Generation of superoxide radicals as a measure of oxidative stress was estimated by flow cytometry. The relative levels of intracellular superoxide were analyzed using the cell permeable redox-sensitive fluorochrome HET. Cells were incubated with 1 µM HET for 15 min at 37°C and subsequently washed twice in cold PBS. The cells were harvested in PBS containing 1% BSA and filtered through 70µM filter and superoxide accumulation was analyzed in terms of increase in ethidium fluorescence using flow cytometry. 10,000 events were collected and the level of superoxide in cells was expressed as percentage of the control (untreated) samples.

Annexin-V staining and FACS analysis

Annexin V binding was used as one measure of apoptotic neurons. Following treatment, both detached and attached cells were harvested and centrifuged. The cell pellet was washed with cold PBS and resuspended in binding buffer containing annexin V-FITC and propidium iodide (PI). The cells were gently vortexed and incubated in the dark. Untreated cells that were either unstained or stained with PI or stained with annexin V-FITC were included along with the experimental samples to correct the background signals. For over expression experiments with adenovirus, annexin V– PE / 7-AAD was used to avoid false positives that could arise as a result
of green fluorescent protein expressed by the bicistronic adenoviral construct. Data were collected on a flow cytometer and analyzed using Cell Quest (BD) software.

**Caspase-glo 3/7 assay**

Caspases-3 and -7 activities were estimated using Caspase-glo 3/7. After treatment, neurons were washed with PBS and 300 µL of caspase-glo 3/7 reagent was added to each well. Cells were scraped and collected in a microfuge tube. The collected lysate was incubated in dark and the resultant luminescence was read in a Glomax luminometer (Promega). RLU was noted and the results were expressed as fold change in caspase 3/7 activity from control.

**Statistical analysis**

Data are presented as means ± s.e.m. Statistical differences were determined using one-way ANOVA when experiments involved more than two groups. Significant differences among the different groups were analyzed using the Student-Newman-Keuls. For the experiments involving only two groups (untreated control and ETOH), a paired student’s t-test was used. A value of \( P<0.05 \) was considered as statistically significant.

**Results**

**Ethanol induces endogenous Nrf2 protein by transcriptional regulation**

Prior studies in our laboratory and others have illustrated that ethanol, both in brain and in cultured neurons elicits oxidative stress (Jacobs and Miller, 2001; Olney et al., 2002; Ramachandran et al., 2001; Ramachandran et al., 2003) a setting that can activate the Nrf2 cytoprotective system (Dong et al., 2008). To determine whether ETOH activates Nrf2 in cerebral cortical neurons (PCN), we initially measured Nrf2 protein levels in PCN treated with ETOH for different periods (Fig. 1A). Immunological detection of Nrf2 using antibody specific against Nrf2 (C-20; sc-722) resulted in the detection of multiple bands and they are marked as 1,
2, 3, 4, 5 in the blot (Fig.1A) corresponding to molecular weights, 100 & 110kDA, 68kDA, 57kDA, 53kDA and 41kDA respectively. Upon Nrf2 silencing using siPORT amine, only the faint 100 & 110 bands and the strong 68kDA band were found to be downregulated while 57, 53 and 41kDA (Fig. 1B) did not show any correspondence to Nrf2 silencing and were not regulated. The transfection agent, siPORT Amine used herein did not have any toxic effect as measured by MTT assay (Supplemental Figure 2). Treatment of PCNs with proteosomal inhibitor, MG-132 were found to increase only 100 & 110 and 68kDA bands in accordance with the previous studies (Furukawa and Xiong, 2005; Kapeta et al., 2010) with the other bands showing no change (Supplemental Figure 3). This suggests that the products detected at 57, 53 and 41kDA could be non-specific. At this point it is not clear whether the ~100kDA observed is native Nrf2 or modified-Nrf2. However, the high molecular weight form could be an anomalous migration of Nrf2 in SDS-PAGE owing to its inherent high acidic charges (Moi et al., 1994). Previous reports by others have also provided evidence that the ~100kDA form is a Nrf2-Actin dimer (Kang et al., 2002), ubiquitinated Nrf2 (Li et al., 2005). Thus, in our experiments, a molecular weight of 68kDA, which is the actual predicted size for Nrf2 is considered. Studies are underway in our laboratory to further identify the nature of both high and low molecular weight Nrf2 forms.

As shown in Fig. 1A, exposure of PCNs to 4mg/ml ETOH resulted in a time-dependent increase in Nrf2 protein levels. This increase, while clearly evident at 6 h time points, ultimately became statistically significant at 12 h of treatment and remained elevated for 24 h (a 2 fold increase). In order to determine whether this induction of Nrf2 protein expression by ETOH is due to an increase in the mRNA levels, total RNA was isolated from PCNs treated with or without ETOH for the same ETOH exposure periods. Fig. 1C illustrates that transcript levels are expressed in a similar pattern as protein although the increase reached statistical significance within 8 h of
treatment. To determine if this reflects an ETOH effect on transcriptional activation of Nrf2, we co-incubated PCNs with and without ETOH and actinomycin D (Act D), which prevents transcription by binding to single-stranded DNA. Shown in Fig. 1D is the effect of Act D on the ETOH-related induction of Nrf2 mRNA. Act D treatment abolished the ETOH-induced Nrf2 mRNA levels at each time point measured and a significant (p<0.05) inhibitory effect was observed as early as 4 h indicating that ETOH regulates Nrf2 by transcriptional mechanisms.

**Ethanol activates Nrf2/ARE binding and nuclear localization in PCN**

To examine whether the above induction of Nrf2 expression in PCN in response to ETOH resulted in functional Nrf2 activity, we assessed Nrf2/ARE DNA binding ability by band shift assay (Fig. 2). In the mobility shift assay with the radiolabeled Nrf2/ARE probe, nuclear extracts obtained from 30 min ETOH treatments showed enhanced ability to form the ARE-DNA protein complex (Fig. 2A, lanes 1 vs 2). A progressive decline was noted at subsequent time intervals. To confirm the specificity of consensus Nrf2/ARE oligonucleotide, we performed gel shift assays in the presence of mutant and unlabelled, cold competitor along with 30 min ETOH nuclear extracts (Fig. 2B). As expected, binding competition by Nrf2/ARE cold probe significantly decreased labeled ARE DNA-protein complex (Fig. 2B, lanes 3 vs 2) while the binding of the latter was unaltered when incubated with ARE core mutated oligo (Fig. 2B, lanes 4 vs 2) confirming the specificity of complex formation. To detect the presence of Nrf2 in this complex formation, we performed supershift analysis using Nrf2 antibody (sc-722). We observed enhanced complex signal instead of an expected supershifted band (Fig. 2B, lanes 6 vs 2). Repeated attempts failed to generate any shift and similar limitations with the use of this Nrf2 antibody (sc-722) have been reported by (Cho et al., 2002). Subsequent supershift assays were performed using biotin-based EMSA with multiple newly available Nrf2 antibodies, out of
which anti-Nrf2 (Origene, TA303616) produced a supershift (Supplemental Figure 4). Additionally, TransAm ELISA for Nrf2 was performed to validate the Nrf2 specific binding. As shown in Fig. 2C, a statistically significant 175% increase in Nrf2 binding activity was found in the nuclear extracts within 30 minutes of ETOH treatment. Prolonged ETOH treatment resulted in a similar decline in Nrf2 activity as seen in Fig 2A. ELISA studies were tightly controlled for antibody and Nrf2/ARE oligo specificity.

As oxidants and xenobiotics activate Nrf2-related systems by promoting nuclear transport (Itoh et al., 1999) we determined whether ETOH induced Nrf2 activity as shown by EMSA and ELISA, reflected nuclear Nrf2 localization. A dual immunofluorescence study showed Nrf2 (stained red) distribution throughout the MAP2 positive neurons (stained green) including cytoplasm and nucleus in untreated control (Fig. 2D). Treatment of ETOH for 30 min resulted in accumulation of Nrf2 in the nuclear compartment (Fig. 2D; compare Control vs ETOH merge image). Further 24 h ETOH treatment significantly upregulated the mRNA level of one of the Nrf2-regulated gene products, NQO1 (Fig. 2E). Altogether, these results demonstrate a role for ETOH in Nrf2 activation and promoting nuclear localization in primary cortical neurons.

**In utero binge ethanol exposure activates Nrf2 in fetal brain cortices**

Having shown that ETOH upregulates Nrf2 expression *in vitro* in PCNs, we next extended the *in vitro* findings to the *in vivo* setting by testing for Nrf2 expression and activation in an animal model of acute ethanol exposure that mimics binge drinking in humans. First, Nrf2 protein expression was determined by western blot in total cortical protein extracts from day 19 fetal brains whose dams were intubated (gastric) with either isocaloric dextrose or ETOH. As Fig. 3A shows, cortical Nrf2 protein expression was increased by almost 3 fold in ETOH exposed group when compared to isocaloric dextrose administered control (P<0.05). Real time PCR analysis
for Nrf2 mRNA illustrated an ETOH-mediated increase in the Nrf2 transcript suggesting that the upregulation of Nrf2 protein is due to increased mRNA levels as occurs in primary cultures of cerebral cortical neurons. *In utero* exposure of ETOH elicited a significant increase (P<0.05) in Nrf2 transcript levels by about 1.5 fold (Fig. 3B). Altered Nrf2 localization associated with its defective signaling may play a critical role in oxidative stress related experimental and clinical neurological diseases such as Alzheimer, Parkinson disease (Ramsey et al., 2007). Hence, western blot analyses for Nrf2 in both cytoplasmic and nuclear enriched fractions from fetal brain cortices were performed (Fig. 3C). GAPDH and Lamin B1 were included as markers for cytoplasmic and nuclear fractions respectively and Nrf2 levels in each fraction were normalized to these. ETOH administration strongly increased endogenous Nrf2 expression when compared to controls (p<0.05). Primarily, this occurred in the nuclear compartment, but cytoplasmic Nrf2 expression was also increased. Fig. 3D is a measure of Nrf2 binding activity by TransAM ELISA for Nrf2 in the nuclear fractions. Activated Nrf2 in nuclear extracts from ETOH treated animals was significantly increased (25%; P<0.05) compared with dextrose administered animals (lane 1 vs lane 2; Fig 3D). To determine specificity of binding, ETOH treated nuclear extracts were incubated with either wild-type Nrf2 oligonucleotide or normal rabbit IgG or mutated Nrf2 oligonucleotide. This illustrated that Nrf2 DNA binding activity was reduced in wild-type and IgG incubated samples (lane 4, 5 vs lane 2; Fig. 3D) whereas the mutated Nrf2 oligonucleotide did not change the Nrf2-DNA binding activity (lane 3 vs lane 2). RT-PCR analysis using primers that specifically amplified the NQO1 gene, a well known Nrf2-transcriptional target revealed that the NQO1 transcript was significantly increased by ~20% in ETOH treated fetal brain cortices when compared with controls (Fig 3E). These results suggest that *in utero* ETOH
administration resulted in upregulation of Nrf2 and its activation in brain cortex of fetuses was similar to those observed in in vitro cortical neuron cultures.

**Nrf2 silencing increases sensitivity to ETOH-related oxidative stress and apoptosis in PCN**

The significance of perturbations of Nrf2 expression on ethanol-related damage to neurons was addressed using siRNA based Nrf2 knockdown experiments. Nrf2 content of PCN was reduced by treatment with 100 nM Nrf2 smartpool siRNA to target endogenous Nrf2 and a non-targeting scrambled siRNA pool to show that silencing of Nrf2 is specific and not due to non-specific effects. siRNA targeting of Nrf2 remarkably reduces the endogenous Nrf2 protein expression in PCNs (lane 3 vs lane 1; Fig. 4A) and this knockdown further abolished the ETOH-induced Nrf2 expression (lane 4 vs lane 2; Fig. 4A). Nrf2/ARE activation regulates cellular glutathione (GSH) homeostasis machinery (Shih et al., 2005a) and ethanol-mediated apoptotic death is dependent on this antioxidant system (Dong et al., 2008). Fig. 4B illustrates that reduced Nrf2 expression decreases baseline GSH content (22%; col 1 vs col 3) and this is further amplified by 44% (col. 3 vs col. 4) upon ETOH treatment as assessed by reduction in MCB fluorescence. Concomitant with this response is a significant increase in superoxide radical levels by 18% in siNrf2 to 27% with ETOH treated Nrf2 depleted neurons (Fig. 4C). Lipid hydroperoxide levels, a measure of lipid peroxidation were also significantly increased in Nrf2 compromised neurons (Supplemental Figure 6A). The effect of this response on ETOH-induced apoptosis is reflected by two apoptotic markers, caspase 3/7 activation and annexin V binding. The reduction in Nrf2 expression further increased (p<0.05) caspase 3/7 activity by 1.5 fold (Fig. 4D) and annexin V binding by 1.2 fold (Fig. 4E) over that occurring in "normal" PCN.

**Overexpression of Nrf2 rendered neuroprotection against ETOH toxicity**

The above findings (Fig. 4) support an important role for Nrf2 in protection of neurons in the
developing brain from ETOH-induced oxidative stress and subsequent apoptotic death. Thus, a key issue is that although ETOH exposure increases Nrf2 protein expression and subsequent early DNA/ARE binding (Figs. 1-3), a portion of these neurons will develop oxidative stress that ultimately elicits apoptotic death (Maffi et al., 2008), (Fig.4). One explanation for this is that the basal Nrf2/ARE cytoprotective machinery in some neurons is incapable of a response that is sufficient to provide protection from ETOH-mediated oxidative stress. It is to be noted that several mechanisms have been reported to be the cause for FASD phenotypes in vivo and elicit toxic responses in cultured neuron models, which include modulation of retinoic acid signaling (Deltour et al., 1996), induction of oxidative stress (Ramachandran et al., 2001; Ramachandran et al., 2003) and calcium based signaling events (Fischer et al., 2003), etc. Herein experiments were performed to address the role of Nrf2 / ARE pathway in regulating ETOH-induced oxidative stress mediated neuronal injury.

**AdNrf2 increased Nrf2 protein and gamma-glutamylcysteine ligase content of PCNs**

PCNs were infected either with replication-incompetent adenovirus specific for Nrf2 (Ad Nrf2), a dominant negative Nrf2 (Ad DN Nrf2), or a control virus (Ad GFP) on 4DIV with a titer of 200 MOI based on the previous study (Shih et al., 2005a; Shih et al., 2003; Shih et al., 2005b). This titer did not cause significant neuronal cell death when compared with virus free conditions (uninfected control PCNs) as measured by MTT (Supplemental Figure 5) and FACS analysis for Annexin V/PE (data not shown). Nrf2 overexpression was confirmed at 24 h and 48 h postinfection by determinations of Nrf2 protein levels. Fig. 5A illustrates that in PCNs infected with Ad GFP, there was only a background low basal level of Nrf2 protein, while expression of Nrf2 in neurons infected with Ad Nrf2 the protein expression increased in a time-dependent manner (lane 2, 3 vs lane 1; Fig. 5A). Overexpression of DN-Nrf2, which lacks an N-terminal
transactivating domain was detected as a 29kDa immunoreactive band in western detection (Fig. 5B) using the same C-terminal Nrf2 antibody that was used to detect full length Nrf2 (Shih et al., 2003). To address the ultimate effectiveness of the Nrf2 overexpression, its impact on neuron content of a major determinant of GSH synthesis capacity, gamma-glutamylcysteine ligase (GCLC) mRNA (Yang et al., 2005) was determined. Ad Nrf2 overexpression resulted in a 3 fold increase in GCLC mRNA expression when compared to Ad GFP infected control (col. 3 vs col. 1; Fig. 5C). Ad Nrf2 infected PCNs when treated with ETOH showed a further enhancement in GCLC mRNA by about 50% (p<0.05) when compared to Ad Nrf2 alone infected PCNs (col. 4 vs col. 3; Fig. 5C). Neither the DN Nrf2 alone infected PCNs nor the ETOH exposed DN Nrf2 infected PCNs showed an increase in GCLC transcript levels (Fig. 5C).

**Nrf2 overexpression prevented the ETOH-mediated decrease in PCN GSH content**

Previous findings have illustrated that preventing the ETOH-related reduction of GSH in PCN mitigates subsequent cell death and that GSH heterogeneity in a single cell type can dictate PCN sensitivity to ETOH (Maffi et al., 2008; Ramachandran et al., 2003; Watts et al., 2005). Cultured PCN express a striking heterogeneity of GSH content and overexpression of Nrf2 clearly influences ETOH induced changes in this GSH heterogeneity (Fig. 5D). Flow cytometric segregation of PCNs based on MCB positivity illustrates three populations of cells that are classified as high, medium and low GSH.

The mean distribution of high, medium and low GSH in Ad GFP infected control cells is 1.1%, 28.7% and 70.2% respectively (col. 1; Fig. 5E). Treatment with ETOH resulted in a clear (p<0.05) shift in GSH distribution which presents as a decrease in both medium GSH (area under green peak; Ad GFP vs Ad GFP+ETOH; Figs. 5D, 5E) and high GSH population (area under blue peak; Ad GFP vs Ad GFP+ETOH; Figs. 5D, 5E) and a shift to the low GSH population.
(area under orange peak; Ad GFP vs Ad GFP+ETOH; Figs. 5D, 5E). By considering the high GSH population (which is 1.1% of the total) as 100%, Nrf2 augmented neurons showed a 20% increase when compared with Ad GFP control. This significantly prevented ETOH induced depletion of high and medium GSH. In contrast to overexpression of wild type Nrf2, use of DN Nrf2 which would be expected to mimic Nrf2 knockdown, likewise showed a dramatic increase in low GSH population with a concomitant decline in the medium and high GSH populations (Fig. 5D). Although glutathione-S-transferase (GST) activity is required for the measurement of intracellular GSH using MCB detection, it has been shown that MCB fluorescence intensity versus time peak labeling in different cell types viz. meninges, astrocytes and differentiated cortical layer II neurons occurs within 20 min. In other words, the reaction between MCB and GSH proceed to completion within 20 min (Sun et al., 2006). Thus the protocol used in our study measures the plateau level of GSH-MCB (30 min MCB incubation; refer materials and methods) ensuring completion of initial GST-mediated conjugation so as to detect the GSH-MCB fluorescence resulting from the cellular GSH concentrations thereby not allowing GST activity as a limiting factor.

**Nrf2 overexpression prevented ETOH-mediated oxidative stress and apoptosis of PCN**

Prior studies by our laboratory and others have connected ETOH-mediated apoptotic death of neurons to oxidative stress (Jacobs and Miller, 2001; Olney et al., 2002; Ramachandran et al., 2001; Watts et al., 2005). FACS analysis was utilized with dihydroethidium (HET) as an estimate of superoxide radical content. ETOH increased the HET signal (Ad GFP + ETOH vs Ad GFP; Fig. 5F) which was normalized in Nrf2 overexpressing neurons (Ad Nrf2 + ETOH vs Ad GFP + ETOH; Fig. 5F). Conversely, in Nrf2 compromised neurons, a robust increase in superoxide generation occurred (shift in peak; Fig. 5F). Associated with normalization of
superoxide levels, the lipid hydroperoxide levels were also attenuated in Nrf2 overexpressing neurons (Supplemental Figure 6B). Both caspase3/7 activation and annexin V binding were utilized as evidence of ETOH-mediate apoptotic death of PCN. Augmentation of Nrf2 significantly prevented the ETOH induced caspase 3/7 activity (col. 4 vs col. 2; Fig. 5G) and annexin V binding to neurons (col. 4 vs col. 2; Fig. 5H). The above findings illustrate that Nrf2 overexpression can mitigate the ETOH induced oxidative stress and prevent the associated neuronal injury.

Discussion:

A major determinant of ETOH’s neurotoxicity is depletion of neuronal GSH content, (Maffi et al., 2008; Watts et al., 2005) and modulation of the Nrf2/ARE/GSH pathways (Yang et al., 2005) has been shown to prevent ethanol induced liver injury in mice (Gong and Cederbaum, 2006). In our current study, we assessed the involvement of Nrf2 and the effect of heterologous Nrf2 overexpression in regulating GSH mediated protection against ETOH in fetal neurons.

Up-regulation of Nrf2 by ethanol in cultured fetal cerebral cortical neurons

In vitro exposure of PCNs to ETOH (4mg/ml; 24h) resulted in a significant increase in both Nrf2 message and protein which is in agreement with previous findings that oxidative stress increases Nrf2 levels and the resultant endogenous antioxidant system (Zhang et al., 2006). In the absence of alcohol, Act D, a potent inhibitor of RNA polymerase II-dependent transcription did not alter Nrf2 mRNA levels while Act D co-incubation with ETOH resulted in a strong decrease in Nrf2 mRNA levels. This illustrates that the observed ETOH-related increased Nrf2 mRNA is newly synthesized e.g. a transcriptionally favored regulation. Of interest, the current Nrf2 regulation paradigm is mainly attributed to post-translational mechanisms (Kang et al., 2010; Purdom-Dickinson et al., 2007). If regulation at the level of stability is playing a role, then co-incubation
of ETOH + Act D could have resulted in the maintenance of high levels of Nrf2 mRNA transcripts when compared to Act D alone treated groups. In line with our findings, Nrf2 mRNA was reported to be upregulated in livers and hepatocytes of chronic alcohol fed rats (Gong and Cederbaum, 2006).

The early increase in nuclear accumulation (Fig. 2D) and DNA-binding of Nrf2 (Fig. 2A, 2C) could be attributed largely to the cytoplasmic release of Nrf2 from Keap1 which could result from redox-related modifications of Keap-1 cysteines and/or phosphorylation of Nrf2 by kinases such as PKC, PI3K, MAPK (Huang et al., 2002; Kang et al., 2002; Yu et al., 1999).

**Ethanol up-regulates Nrf2 in the intact fetal cerebral cortex**

The *in utero* binge model demonstrated that the increased Nrf2 expression combined with functional activation in brain cortices of fetuses supported its congruity to *in vitro* cortical neuronal cultures. Our findings are consistent with a previous study showing that maternal ETOH exposure increases Nrf2 expression, ARE binding/signaling pathways in mouse embryos (Dong et al., 2008). Notably, the transcriptional activation of Nrf2 observed in response to ETOH *in vitro* at 30 min (increase) and at 24 h (little to none) is not mirrored by *in utero* observations (which were determined at the end of the treatment period). In general, temporal effects on transcriptional activity seen *in vitro* in cellular systems cannot be directly correlated to *in vivo* observations. In our studies, these deviations may be accounted for by: (1) the difference in dosing patterns between the two experimental settings, *in vitro* with a single high dose of ETOH and *in utero* with a well documented binge exposure which uses multiple doses administered at 12 h intervals and (2) the possible influences of developmental stage, maternal dependency and physiological environment in *in utero* settings which might differentially impact on binding affinities of respective transcription factors to relevant consensus sites. Although
Nrf2 transcriptional activity did not temporally correlate between the *in utero* and *in vitro* settings, our results illustrate that an event that is occurring *in vitro* in response to ethanol is also occurring *in utero* with a pattern of maternal ethanol consumption that elicits damage to the developing brain. In response to oxidative stress, Nrf2 accumulates in the nucleus with an associated decrease in cytoplasm. However, prenatal ETOH exposure increased Nrf2 in both cytoplasmic and nuclear fractions (Fig 3C). Though the mechanism underlying this increase is not established, it could be attributed to the newly synthesized Nrf2 mRNA (Fig 3B). In response to tBHQ, a similar increase in Nrf2 expression in both cytoplasm and nucleus was observed (He et al., 2007) and ETOH might activate a similar mechanism to regulate Nrf2’s localization and function.

**Nrf2 targeting mitigates ETOH-induced oxidative stress**

A critical insight from gene specific RNAi based knockdown experiments showed that ETOH induced neurotoxicity is dependent on Nrf2 signaling in cortical neurons. This is reflected by a significant decrease in intracellular GSH content, concomitant with increased levels of superoxide and hydroperoxide (Fig. 4B; 4C; Supplemental Figure 6A). In support of our findings, data obtained in Nrf2 knockout mice and mammalian cells showed that Nrf2 modulates GSH levels by regulating the expression of GCLC, an enzyme involved in GSH biosynthesis (Yang et al., 2005). Cells deficient in Nrf2 display a disturbed redox homeostasis and are exquisitely sensitive to various oxidants as reviewed (Kensler et al., 2007). This shows that Nrf2 could serve as a key control point in both basal and ETOH induced oxidative defenses.

**Ethanol elicits neuron death in the presence of its Nrf2 up-regulation**

Although ETOH enhanced Nrf2 expression both *in vitro* and *in utero*, neuron survival was still impaired (Ramachandran et al., 2001; Ramachandran et al., 2003). A reasonable explanation for
this is that the ETOH-related Nrf2 up-regulation is simply insufficient to protect. Other plausible reasons for circumvention of Nrf2/antioxidant protection during prolonged ETOH treatment (24 h) include nuclear exclusion of Nrf2, direct caspase activation and persistent ROS generation (current study), (Ramachandran et al., 2003). Nevertheless, the increases in Nrf2 may reflect a critical adaptive response to counteract the ETOH-induced oxidative stress and an effort to maintain redox homeostasis. Consistent with our findings, (Hirota et al., 2005) demonstrated that UVA irradiation, triggered Nrf2 expression and activation, however this failed to limit apoptosis in fibroblasts. The exaggerated caspase 3/7 activity and apoptosis (Fig 4D, Fig 4E) seen in ETOH treated Nrf2 deficient neurons could be ascribed to the enhanced accumulation of superoxide radical (Fig. 4C) and a subsequent loss in GSH levels (Fig. 4B). Thus, our results reveal that there is a definitive role for Nrf2 in ETOH-induced apoptosis and the deficiency of Nrf2 could sensitize fetal cortical neurons to the pro-apoptotic effects of ETOH. Although a definitive role for Nrf2 is evident, a tight quantitative correlation between manipulations of Nrf2 expression (siNrf2 knockdown) and subsequent neuroprotection (apoptosis measures) is not achieved in our experimental conditions. The contribution from residual Nrf2 towards survival signals could explain this. Importantly, it has been demonstrated that overexpression of Nrf2 was not able to prevent death of neurons treated with staurosporine, a potent inhibitor of phospholipid/Ca\textsuperscript{2+}-dependent protein kinase (Shih et al., 2003) suggesting that modulation of Nrf2 may not necessarily protect neurons from apoptosis induced by certain pathways which are not involving ROS. Overall our results suggest that there is an explicit involvement of Nrf2 in ETOH-mediated apoptosis, however the influence of Nrf2-independent events cannot be ruled out.

**Overexpression of Nrf2 thwarts ETOH-induced apoptosis in fetal cortical neurons**
Under basal condition, two molecules of Keap1 sequester one molecule of Nrf2 in the cytoplasm and target Nrf2 for proteasomal degradation (Wakabayashi et al., 2004), leaving less Nrf2 molecules compared to Keap1. Although ETOH induces endogenous Nrf2 and activates defense, this increase may not be sufficient to outweigh Keap1 levels which in turn can keep Nrf2 under control. Very recently, tBHQ treatment, a known inducer of Nrf2 has been shown to override ETOH-induced oxidative stress and prevent neural crest cell apoptosis (Yan et al., 2010). Herein, we adopted a direct upregulation strategy by heterologous overexpression of Nrf2, with the intent of overwhelming the binding capacity of Keap1 and freeing Nrf2 molecules to activate ARE-dependent genes before ETOH insult. A previous study demonstrated that direct Nrf2 overexpression resulted in robust ARE-dependent transcription and cytoprotection (Shih et al., 2005b). Further, as DNA based transfection of primary neurons in culture can be experimentally challenging, we adopted a well suited and efficient adenoviral strategy to overexpress Nrf2. In Nrf2 overexpressed condition, the GSH depleting effects of ETOH (Fig. 5D, 5E) appeared to be compensated by an increase in the rate-limiting enzyme in GSH synthesis namely GCLC (Fig 5C). However, the ethanol-related increase in Nrf2 expression did not generate a significant up-regulation of GCLC. One potential explanation is as follows: ETOH, a complex toxin is known to increase TGF beta 1 in neurons (Kuhn and Sarkar, 2008) and the latter is known to decrease GCLC mRNA (Franklin et al., 2003). Thus, ETOH could stimulate both GCLC mRNA synthesis (by increasing Nrf2) as well as GCLC downregulation by a TGF beta 1 mediated mechanism, thereby mitigating a significant increase in the net copies of GCLC mRNA. This would not happen with adenovirus-mediated Nrf2 overexpression.

Supporting the importance of Nrf2 in neuroprotection from ETOH, is the increased sensitivity to this compound associated with dysregulation of GSH (Ramachandran et al., 2003). A functional
consequence of this is a selective vulnerability of cerebral cortical neurons to ETOH which is dependent on heterogeneity of GSH homeostasis (Maffi et al., 2008). The present study supports our earlier findings that, a 24 h ETOH exposure dramatically reduced the high and medium GSH populations, (Fig. 5D, 5E) along with increasing superoxide (Fig. 5F), hydroperoxide (Supplemental Figure 6A), caspase 3/7 activity (Fig. 5G) and apoptosis (Fig. 5H). All of the aforementioned events were corrected by Nrf2 overexpression thus helping in maintenance of intracellular redox balance. In accordance with our results, up-regulation of Nrf2 was correlated to increased intracellular GSH, decreased caspase 3 activity rendering neuroprotection against various oxidative stressors in various models including neuron-glia (Cho et al., 2002; Dong et al., 2008; Kraft et al., 2004; Li et al., 2005; Shih et al., 2003). It remains to be determined whether differential Nrf2 levels likewise exist in neurons with such a scenario being responsible for phenotypic divergences in GSH homeostasis and sensitivity to toxins.

In conclusion, our current studies demonstrate that while ETOH transcriptionally induces Nrf2 activation as a potentially protective stress response, this response is insufficient to prevent damage to select populations of these cells. Yet, the importance of the neuron Nrf2-mediated cytoprotection systems is clearly illustrated by multiple loss and gain of function experiments. The functional relevance of this to ETOH-related damage to the developing brain is that the periods of highest sensitivity to ethanol are the second and early third trimester equivalents during which there is little to no astrocyte-mediated maintenance of GSH homeostasis. Thus, interventions to provide protection of neurons at these sensitive developmental stages must be at the neuron level.
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Authorship Contributions

Participated in research design : M. Narasimhan, L. Mahimainathan, G. I. Henderson

Conducted experiments : M. Narasimhan, L. Mahimainathan, M. Rathinam, A. Riar

Performed data analysis : M. Narasimhan, L. Mahimainathan, M. Rathinam

Wrote or contributed to the writing of manuscript : M. Narasimhan, L. Mahimainathan, G.I. Henderson
References


post-translational and caspase-independent transcriptional regulatory mechanisms. *FASEB J* 17(11):1535-1537.


Foot notes

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MN and LM contributed equally to this work.

Name and address of person to receive reprint request:

George I Henderson, Ph. D. (or)

Lenin Mahimainathan, Ph. D.

Department of Pharmacology and Neuroscience; South Plains Alcohol and Addiction Research Center (SPAARC)

Texas Tech University Health Sciences Center

3601 4th Street, STOP 6592 , Lubbock, Texas, 79430, USA

Tel: 806-743-2425

Fax: 806-743-2744

George I Henderson; E-mail: george.henderson@ttuhsc.edu

Lenin Mahimainathan; E-mail: lenin.mahimainathan@ttuhsc.edu
Figure Legends

Figure 1. Ethanol induces Nrf2 expression in a time-dependent manner in primary cortical Neurons. PCNs were treated with ETOH (4mg/ml) for the times indicated. (A) Nrf2 protein expression was determined by immunoblot analyses and equal loading demonstrated by anti-GAPDH. Lower panel depicts densitometric scanning analysis ratio of Nrf2 to GAPDH. The superscript $^{(1)}$ & $^{(2)}$ in the densitometric plot represents the ratio intensity of 100-110 & 68 kDA products corrected to GAPDH respectively (B) PCNs were transfected with either non-targeting scramble siRNA or smart pool mix of four siNrf2 using siPORT amine. Cells were lysed 48 h post transfection and immunoblot analysed with anti-Nrf2 and GAPDH. Lower panel depicts densitometric scanning analysis ratio of 68 kDA Nrf2 to GAPDH. (C) PCNs were treated with ETOH (4mg/ml) for the times indicated and real time qPCR (RT qPCR) analysis for Nrf2 transcript expression normalized to GAPDH. (D) Cells were incubated with Act D (1µg/ml) along with ETOH (4mg/ml) for given time in hours. Total RNA was then extracted and subjected to RT qPCR for Nrf2 and GAPDH. (A-C) The results are the mean ± s.e.m. of experiments performed in triplicate. One way ANOVA for sub-panel (A, C, D) and t-test for sub-panel (B) was performed to establish statistical significance. In A, C $^{*}P<0.05$ and $ns$-not significant compared with untreated control. In B, $^{*}P<0.05$ - compared with untreated control. In D $^{*}P<0.05$ - compared with untreated control; $ns$ -not significant compared with untreated control and $^{#} P<0.05$ – compared with respective ETOH time treatment.

Figure 2. Ethanol induces Nrf2/ARE binding activity in PCN nuclear extracts. (A) Nuclear protein obtained from control and PCNs exposed to ETOH (4mg/ml) for different time points were used to detect ARE-specific oligonucleotide–protein complexes by EMSA (B) Supershift analysis was performed using Nrf2 antibody and assayed for presence of Nrf2 in the ARE DNA-
protein complex. Excess unlabeled (cold) and mutant oligonucleotide was used to determine specificity of Nrf2 / ARE binding. (C) Trans AM ELISA for Nrf2 was performed to confirm Nrf2 DNA binding activity in control and ETOH treated PCN nuclear extracts (mean ± s.e.m, n=3). Statistical significance was established by one way ANOVA. *P<0.05 - compared with untreated control; ns -not significant compared with untreated control (D) A representative immunofluorescence photomicrograph (20X) from control and 30 min ETOH (4mg/ml) treated PCNs for Nrf2 nuclear localization. Anti-MAP2 was used as neuronal marker and the nucleus was identified by DAPI staining. Data are representative of three independent experiments. (E) RT-PCR image and statistical analysis for NQO1 and GAPDH mRNA from PCN treated with and without ETOH (4mg/ml) (n=4); *P<0.05 - compared with untreated control.

Figure 3. Prenatal ethanol exposure increases Nrf2 expression and activation in fetal brain cortices. Pregnant rats (Sprague-Dawley) at embryonic day 17 (E17) were administered ETOH (4g/kg body weight) or isocaloric dextrose by gastric intubation at 12 h intervals for two days. At E19 brain cortex from embryos were dissected and processed for Nrf2 protein expression by immunoblotting (A, n=6), Nrf2 mRNA expression by RT qPCR (B, n=6). Lower panel in A depicts densitometric scanning ratio of Nrf2/ GAPDH intensities. In A, B Student’s t test (paired) was performed to determine the significance of treatment. *P<0.05 compared with isocaloric dextrose administered animals. Nuclear and cytosolic extracts prepared were employed for immunoblot analyses of Nrf2 to determine localization (C, mean ± s.e.m, n=5) and Nuclear extracts were used in Trans AM ELISA for Nrf2 activity determination (D, mean ± s.e.m, n=3) One way ANOVA indicates *P<0.05, ns -not significant when compared with isocaloric dextrose administered animals. (E) RT-PCR image and statistical analysis for NQO1 and
GAPDH mRNA from control and ETOH treated fetal brain cortices (n=4); *P<0.05 - compared with isocaloric dextrose treated control.

**Figure 4. siRNA mediated Nrf2 knockdown exacerbates ethanol induced oxidative stress and neuronal death.** (A) PCNs were transfected with either non-targeting scramble siRNA or smart pool mix of four siNrf2 using siPORT amine. 24 h post transfection of scrambled siRNA or siNrf2 the cells were treated with or without ETOH (4mg/ml) for additional 24 h. Protein extracts were then immunoblot analysed with anti-Nrf2 and GAPDH. (B) Live cells were loaded with MCB and cells were fluorimetrically analysed for GSH content and data is presented as relative fluorescence units (mean ± s.e.m, n=6). In panel C, live cells were loaded with HET followed by FACS sorting for ethidium positivity (measure of superoxide) to indicate level of oxidative stress (mean ± s.e.m, n=6). In panel D cell extracts were used in Caspase-Glo assay and caspase3/7 activity was determined as luminescence units (mean ± s.e.m, n=6). In panel E live cells were stained using Annexin V- FITC conjugate / PI and FACS sorted to measure apoptotic cell death (mean ± s.e.m, n=6). One way ANOVA was performed to establish statistical significance. *, @ P<0.05 - compared with scramble untreated control; # P<0.05 - compared with siNrf2.

**Figure 5. Adenovirus mediated overexpression of Nrf2 contained ethanol-induced oxidative stress and neuronal death.** (A) PCNs (4 days *in vitro* (DIV)) in serum containing media were infected with adenovirus encoding Nrf2 cDNA (A) or DN Nrf2 (B) at 200 multiplicity of infection (MOI). 24 and 48 h post-infection cells were processed for protein and immunoblot analysed for Nrf2 overexpression and normalized with anti-Tubulin/GAPDH expression. Adenovirus encoding GFP cDNA was used as control virus in this study. (C-J) PCNs were infected with either Ad GFP/ Ad Nrf2/ Ad DN Nrf2 for 24 h at 4 DIV and followed by 24 h of
ETOH (4mg/ml) treatment. (C) Total RNA was extracted and subjected to RT qPCR for GCLC, an Nrf2 transcriptional target and a constitutively expressed gene, GAPDH. Relative quantification of GCLC/GAPDH transcripts is illustrated (mean ± s.e.m, n=6). (D) Neurons infected and treated as in panel C were subjected to flow cytometric determination of MCB staining to measure cellular GSH. A representative experiment is shown (D) wherein neuronal population were divided into low (orange), medium(green) and high GSH (blue) containing cells based on differential MCB staining. Panels E depict percentage of cells positive for MCB derived from high, medium and low GSH population represented in blue, green and orange graphs respectively (mean ± s.e.m, n=6). Panel F is a representative FACS diagram, portraying the effect of Nrf2 on ethanol induced superoxide generation measured in terms of oxidation of hydroethidine (HET) into the fluorescent product ethidium. Red vertical bars in panels are introduced to visualize shift in peaks. Panel G depicts the estimation of effector caspase3/7 activity in neuronal extracts obtained from aforementioned treatment by Caspase-Glo assay (mean ± s.e.m, n=4). In panel H, neurons were stained with Annexin V-PE conjugate/7-Amino-Actinomycin (7-AAD) and read immediately by flow cytometry to measure the extent of apoptosis (mean ± s.e.m, n=6). One way ANOVA was performed to establish statistical significance. *, @ P<0.05 vs AdGFP control; # P<0.05 vs AdGFP + ETOH; ns - not significant.
Fig. 1

A

**Time (h)** 0 6 12 24

**ETOH** - + + +

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**Nrf2 (C-20)**

**GAPDH**

**Ratio of Intensity**

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- ns
- *
- ETOH +++-

**Molecular Pharmacology Fast Forward. Published on August 26, 2011 as DOI: 10.1124/mol.111.073262**

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Fig. 1

B

Scr. siRNA + -

siNrf2 - +

kDa

150

100

75

50

37

Nrf2 (C-20)

GAPDH

Relative Intensity Nrf2/GAPDH

0.8

0.4

0.0

*
Fig. 1
Fig. 1

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Fig. 2

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ARE DNA-Protein complex
Fig. 2

**B**

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ARE DNA-Nrf2 complex
Fig. 2

The figure shows the percentage change in Nrf2 activity from control over time (0, 0.5, 1, 2, 4, 6, 24, 0.5, 0.5 hours) for different treatments: ETOH, Mutant, Wt competitor, and IgG. The figure indicates that ETOH had a significant effect on Nrf2 activity at various time points, while Mutant and Wt competitor showed no significant change. IgG treatment also had no significant effect. Statistical significance is indicated by asterisks (*), and ns denotes no significant change.
Fig. 2

D

**CONTROL**

Nrf2 | MAP2 | Merge
---|---|---

**ETOH**

Nrf2 | MAP2 | Merge
---|---|---

This article has not been copyedited and formatted. The final version may differ from this version.
**Fig. 2**

![Diagram showing relative intensity of NQO1 and GAPDH with and without ETOH.](image)

**Relative Intensity NQO1/GAPDH**

- **ETOH -**
- **ETOH +**

* indicates a statistically significant difference.
Fig. 3

A

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Relative intensity Nrf2/GAPDH

*
Fig. 3

B

![Bar graph showing relative quantification of Nrf2/GAPDH with and without ETOH treatment.](image-url)
Fig. 3

C

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Fig. 3

D

% change in Nrf2 activity from control

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<td>Control</td>
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<td>-</td>
</tr>
<tr>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01
Fig. 3

E

NQO1

GAPDH

Relative Intensity
NQO1/GAPDH

ETOH - +

*
Fig. 4

A

<table>
<thead>
<tr>
<th></th>
<th>ETOH</th>
<th>Scramble</th>
<th>siNrf2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ETOH</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Scramble</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>siNrf2</td>
<td></td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

![Nrf2]

![GAPDH]
Fig. 4

![Graph showing the effect of ETOH, Scramble, and siNrf2 on RFU with symbols indicating statistical significance]

- ETOH: - + - +
- Scramble: + + - -
- siNrf2: - - + +

RFU: 0, 25000, 50000, 75000, 100000, 125000

Symbols used:
- *= significant difference
- # = significant difference
- @ = significant difference
Fig. 4

C

% HE positive cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>ETOH</th>
<th>Scramble</th>
<th>siNrf2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ETOH</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>siNrf2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* @ #
Fig. 4

**Fold change in caspase 3/7 activity**

<table>
<thead>
<tr>
<th></th>
<th>ETOH</th>
<th>Scramble</th>
<th>siNrf2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- ETOH: - = -fold, + = +fold
- Scramble: + + = +fold, + - = -fold
- siNrf2: - - = -fold, + + = +fold

* = p < 0.05
@ = p < 0.01
# = p < 0.001
Fig. 4

E

% change in annexin positive cells

<table>
<thead>
<tr>
<th></th>
<th>ETOH</th>
<th>Scramble</th>
<th>siNrf2</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
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<td>+</td>
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<tr>
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<td></td>
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</tr>
</tbody>
</table>
Fig. 5

A

<table>
<thead>
<tr>
<th>AdGFP</th>
<th>+</th>
<th>-</th>
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</thead>
<tbody>
<tr>
<td>WT Nrf2</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

48h 24h 48h

Nrf2

Tubulin
Fig. 5

<table>
<thead>
<tr>
<th></th>
<th>AdGFP</th>
<th>Ad DN Nrf2</th>
<th>48h</th>
<th>48h</th>
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</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- 29kDA
- Nrf2DN
- GAPDH
**Fig. 5**

<table>
<thead>
<tr>
<th>Condition</th>
<th>ETOH</th>
<th>AdGFP</th>
<th>Ad WT Nrf2</th>
<th>Ad DN Nrf2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rel. quantification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCLC/GAPDH</td>
<td>1.0</td>
<td>ns</td>
<td>2.5</td>
<td>ns</td>
</tr>
</tbody>
</table>

- *p < 0.05
- ns = not significant

**Legend:**

- ETOH
- AdGFP
- Ad WT Nrf2
- Ad DN Nrf2
<table>
<thead>
<tr>
<th></th>
<th>AdGFP</th>
<th>AdGFP + ETOH</th>
<th>Ad WT Nrf2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad WT Nrf2 + ETOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad DN Nrf2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad DN Nrf2 + ETOH</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5
Fig. 5

![Bar graphs showing % MCB positive cells for different conditions](image)

- **ETOH**:
  - AdWT Nrf2
  - AdDN Nrf2

- **AdGFP**:
  - ETOH

- **Ad WT Nrf2**:
  - ETOH

- **Ad DN Nrf2**:
  - ETOH

Legend:
- *: p < 0.05
- #: NS
- @: p < 0.01
- #: p < 0.001
- NS: Not significant

% MCB positive cells for different conditions.
Fig. 5

<table>
<thead>
<tr>
<th>ETOH</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdGFP</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ad WT Nrf2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Fold change in caspase 3/7 activity

- 0.0
- 0.5
- 1.0
- 1.5
- 2.0

* ns
# ns
Fig. 5

% change in annexin positive cells

- ETOH
  -
  +

- AdGFP
  +
  +
  -
  -

- Ad WT Nrf2
  -
  -
  +
  +

H

200
150
100
50
0

ns
*
#
ns

50
100
150
200

This article has not been copyedited and formatted. The final version may differ from this version.
Supplemental Data For:

**Article Title**: Overexpression of Nrf2 protects cerebral cortical neurons from ethanol-induced apoptotic death

**Authors**: Madhusudhanan Narasimhan, Lenin Mahimainathan, Mary Latha Rathinam, Amanjot Kaur Riar and George I. Henderson

**Journal**: Molecular Pharmacology

**Manuscript #**: 73262
Supplemental Figure 1: Purity of isolated primary cortical neurons. Dual-immunofluorescence assay was performed using an anti-microtubule associated protein-2 (MAP-2) marker for neurons (Green) and possible contamination by astrocytes was assessed using anti-glial fibrillary acidic protein (GFAP) (Red). The cells were visualised under confocal microscope and images captured using 40x magnification. Occasional one or two GFAP positive cells pointing out astrocyte contamination was observed similar to that shown in figure (white arrow in GFAP and merge panel). Five different fields were randomly counted for GFAP and MAP2 positive cells. Approximately 95% of the cells were neurons.
Supplemental Figure 2: Transfection agent, siPORT Amines did not have any toxicity in PCNs. As indicated in the materials and methods section, PCNs were transfected with scramble siRNA using siPORT amine and assessed for cell death, if any, using MTT assay. Briefly, at the end of 48 h after transfection, the cells were washed with RPMI-1640 phenol red free media and 1.5ml of 1/10 diluted 5mg/ml MTT solution was added to each well and incubated at 370 C for 2 h. MTT solution was then removed and the purple colored formazan complex formed was dissolved using 600 µL isopropanol/HCl/Triton X-100 and measured at a wavelength of 570nm. The toxicity due to transfection agent is represented in terms of percentage change in survival between the non-transfected and transfected cells (n=4).
Supplemental Figure 3: MG-132 treatment stabilizes Nrf2 protein in PCNs. PCNs were treated with 10μM of proteasomal inhibitor, MG-132 for 4 h and western analysis were performed for Nrf2 using C-20 (sc-722) and GAPDH. A representative blot is given.
Supplemental Figure 4: Supershift analysis of ETOH treated PCNs using biotin based EMSA. NFE2 consensus oligonucleotide was end-labeled with biotin as per the instructions provided in the biotin 3’ end DNA labeling kit (Thermo Scientific, Rockford, IL). 10 μg nuclear extracts from untreated and 30 min ETOH treated PCNs were used in the binding reaction containing 3 μL of biotinylated Nrf2 oligo and other constituents for 20 min as mentioned in LightShift chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL). Independently, for supershift analysis, nuclear extract from ETOH treated sample was preincubated for 30 min in ice either with 2.5 μL of rabbit monoclonal antibody against Nrf2 (TA303616, Origene, Rockville, MD) or IgG. This antibody incubated sample was then subsequently subjected to binding reaction along with biotinylated Nrf2 oligo for additional 20 min at RT. These samples were electrophoresed, transferred onto a positively charged Biodyne B nylon membrane (60209, Pall life sciences, Ann Arbor, MI). The membrane was then croslinked at 120mJ/cm² and the biotin-labeled DNA was detected using streptavidin-HRP/ chemiluminescence based autoradiography as mentioned in LightShift chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL).
Supplemental Figure 5: Adenovirus infection did not have any toxicity in PCNs. As indicated in the materials and methods section, PCNs were infected with AdGFP and Ad WT Nrf2 for 48 h and assessed for cell death, if any, using MTT assay. Briefly, at the end of 48 h after infection, the cells were washed with RPMI-1640 phenol red free media and 1.5ml of 1/10 diluted 5mg/ml MTT solution was added to each well and incubated at 37°C for 2 h. MTT solution was then removed and the purple colored formazan complex was dissolved using 600 μL isopropanol/HCl/Triton X-100 and measured at a wavelength of 570nm. The toxicity due to infection is represented in terms of percentage change in survival between the uninfected and adenovirus-infected cells (n=6).
Supplemental Figure 6: Lipid hydroperoxide measurement in Nrf2 knockdown (A) and adenovirus mediated overexpression of Nrf2 (B) in PCNs. As indicated in materials and methods section, PCNs were either downregulated with siRNA specific for Nrf2 or overexpressed using adenovirus encoding Nrf2 for 24 h, treated with ETOH for additional 24 h and assayed for lipid hydroperoxides, a measurement of lipid peroxidation. Briefly, the lysates were collected in HPLC grade water, sonicated and the collected supernatant is subjected to chloroform extraction as recommended in lipid hydroperoxide assay kit (705002, Cayman chemical company, Ann Arbor, MI). An aliquot of the samples were diluted with chloroform-methanol (2:1) to a volume of 950 μL and 50 μL of chromagen was added, incubated for 5 min at RT and read at 500 nm in a spectrophotometer. Non-hydroperoxide generated color in this assay is measured by reducing the hydroperoxides from samples with triphenylphosphine. These values were used to correct for any background absorbance in the samples. Before chloroform extraction, an aliquot of the samples were set aside and protein was estimated. The triphenylphosphine corrected LPO values were finally corrected to protein and represented (n=3). In A, B *P<0.05 compared with untreated control. In A, @ and # P<0.05 – vs control and siNrf2 respectively. In B, $ P<0.05 vs Ad GFP and ns - not significant vs Ad WT Nrf2.