Resveratrol Restores Nrf2 Level and Prevents Ethanol-Induced Toxic Effects in the Cerebellum of a Rodent Model of Fetal Alcohol Spectrum Disorders

Ambirish Kumar, Chandra K. Singh, Holly A. LaVoie, Donald J. DiPette, and Ugra S. Singh

Department of Pathology, Microbiology, and Immunology (A.K., C.K.S., U.S.S.), and Department of Cell Biology and Anatomy (H.L., D.J.D.), University of South Carolina School of Medicine, University of South Carolina, Columbia, South Carolina

Received January 12, 2011; accepted June 21, 2011

ABSTRACT

In humans, ethanol exposure during pregnancy produces a wide range of abnormalities in infants collectively known as fetal alcohol spectrum disorders (FASD). Neuronal malformations in FASD manifest as postnatal behavioral and functional disturbances. The cerebellum is particularly sensitive to ethanol during development. In a rodent model of FASD, high doses of ethanol (blood ethanol concentration 80 mM) induces neuronal cell death in the cerebellum. However, information on potential agent(s) that may protect the cerebellum against the toxic effects of ethanol is lacking. Growing evidence suggests that a polyphenolic compound, resveratrol, has antioxidant and neuroprotective properties. Here we studied whether resveratrol (3,5,4′-trihydroxy-trans-stilbene), a phytoalexin found in red grapes and blueberries, protects the cerebellar granule neurons against ethanol-induced cell death. In the present study, we showed that administration of resveratrol (100 mg/kg) to postnatal day 7 rat pups prevents ethanol-induced apoptosis by scavenging reactive oxygen species in the external granule layer of the cerebellum and increases the survival of cerebellar granule cells. It restores ethanol-induced changes in the level of transcription factor nuclear factor-erythroid derived 2-like 2 (nfe2l2, also known as Nrf2) in the nucleus. This in turn retains the expression and activity of its downstream gene targets such as NADPH quinine oxidoreductase 1 and superoxide dismutase in cerebellum of ethanol-exposed pups. These studies indicate that resveratrol exhibits neuroprotective effects in cerebellum by acting at redox regulating proteins in a rodent model of FASD.

Introduction

Exposure of the developing fetus to ethanol causes various birth defects in humans collectively known as fetal alcohol spectrum disorders (FASD) (Guerri et al., 2009). In the United States, approximately 40,000 children are born with FASD each year, costing an estimated $6 billion in health, education, and social services (May and Gossage, 2001; Wattendorf and Muenke, 2005). Although prenatal ethanol exposure affects the development of almost all organs, the brain and craniofacial features show the most severe defects, and the cerebellum is particularly sensitive to the effects of ethanol during development (Bailey and Sokol, 2008; Guerri et al., 2009). The cerebellar granule neurons (CGNs) undergo apoptosis in response to ethanol exposure (Joshi et al., 2006). Ethanol-induced apoptosis in neurons is caused by reactive oxygen species generated as a result of oxidative stress (Chu et al., 2007; Ikonomidou and Kaindl, 2011).

Neurons are most sensitive to the harmful effects of ethanol during the period of synaptogenesis. During this period (also called brain growth spurt stage), there is increased neuronal differentiation and synapse formation in the developing cerebellum by acting at redox regulating proteins in a rodent model of FASD.
op ing brain. The synaptogenic phase in the human fetus begins during the third trimester of pregnancy and continues for the first few years of postnatal life (Dobbing and Sands, 1979; Olney et al., 2000). This period in rodents corresponds to postnatal days 4 to 9. Exposure of rodents to high-dose ethanol during this period depletes the neurons in the cerebellum (Bonthius and West, 1990; Napper and West, 1995). For these reasons, rodents are an excellent experimental animal model to study the mechanism of FASD. In our previous studies, we have shown ethanol exposure to rats at postnatal day 7 inhibits the differentiation and induces apoptosis in the CGNs (Joshi et al., 2006).

Natural compounds with antioxidative properties are used in the treatment of various diseases. One such compound, resveratrol (3,5,4′-trihydroxy-trans-stilbene), exhibits a wide range of pharmacological effects, including prevention of heart disorders, blocking of lipoprotein oxidation, free radical scavenging, prevention of apoptosis, and inhibition of platelet aggregation (Shakibaei et al., 2009). Because of the strong therapeutic potential, clinical trials for resveratrol in human for various diseases are currently being conducted (http://clinicaltrials.gov/ct2/results?term=resveratrol). Prior studies have shown that resveratrol prevented ethanol-induced apoptosis in embryonic stem cells and ethanol-disrupted mouse blastocyst development (Huang et al., 2007) and also protected ethanol-induced apoptosis in fetal rhombencephalic neurons (Antonio and Druse, 2008). In a recent study, resveratrol was found to improve ethanol-induced cognitive deficits in a rodent model of FASD (Tiwari and Chopra, 2011). Whether resveratrol protects cerebellum and CGNs against the neurotoxic effects of ethanol, as well as the underlying mechanism, is not known.

To determine whether resveratrol prevents ethanol-induced apoptosis, we used a postnatal day 7 rodent model of FASD and demonstrated that ethanol increases oxidative stress and inhibits the activation of Nrf2 transcription factor in the cerebellum and in CGNs. Administration of resveratrol (100 mg/kg) before ethanol exposure restores the level of transcription factor Nrf2, prevents ethanol-induced oxidative stress in the cerebellum, and promotes survival of cerebellar granule cells. These studies show that resveratrol is a potent antioxidant that targets Nrf2 for protecting neurons against neurotoxic effects of ethanol.

Materials and Methods

All animals used in these studies were handled in accordance with national guidelines for animal welfare, and protocols were approved by the University of South Carolina Animal Care Committee. The present study was performed using randomly selected postnatal day 7 Long Evans rat pups (Charles River Laboratories, Inc., Wilmington, MA). All the chemicals were used from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Exposure of Postnatal Rat Pups to Ethanol and Resveratrol Administration

For ethanol exposure, pups along with nursing dams were placed in an inhalation chamber for 5 h as reported previously (Joshi et al., 2006; Kumar et al., 2010). At the end of the inhalation period, we estimated blood alcohol concentration (BAC) with an alcohol reagent kit (Pointe Scientific, Canton, MI) according to the manufacturer’s instructions. Mean BAC values were calculated from BACs obtained from three sets of five pups each. Ethanol exposure according to this protocol produced 80 mM concentration in the blood (Joshi et al., 2006). A total of 226 pups were used for these studies. Pups were divided into 10 groups: ethanol-unexposed, ethanol-unexposed plus resveratrol (2, 20, 40, or 100 mg/kg), ethanol-exposed, and ethanol-exposed plus resveratrol (2, 20, 40, or 100 mg/kg). (Throughout the text, the resveratrol values refer to kilograms of body weight.) Each group included at least four to six pups in individual studies. Resveratrol (Sigma-Aldrich) was suspended in water and administered twice by oral gavage, 24 h and 1 h before ethanol exposure. After ethanol exposure, pups were immediately sacrificed by decapitation, and cerebella were collected for isolation of whole-cell extracts and preparation of CGN cultures.

Isolation and Primary Culture of CGNs

Primary culture of CGNs were prepared from at least six to eight pups per group in individual studies (Bhave and Hoffman, 1997; Joshi et al., 2006; Kumar et al., 2010). In brief, cerebella isolated from different treatment groups immediately after ethanol exposure were enzymatically digested with trypsin (Atlanta Biologicals, Lawrenceville, GA) at 37°C for 20 min and treated with soybean trypsin inhibitor (Worthington, Lakewood, NJ) and DNase I (Worthington) for 5 min at 37°C. Cells were dissociated by trituration, washed once with basal medium Eagle’s containing 25 mM KCl, and plated in basal medium Eagle’s containing 25 mM KCl, 10% fetal bovine serum, and 1% antibiotic solution. To prevent growth of non-neuronal cells, 10 μM cytosine-d-arabinofuranoside (Sigma-Aldrich) was added to the cultures after 12 to 14 h. With this protocol, nearly 95% of the cells were CGNs.

Identification of Total Cerebellar Proteins by Western Blot Analysis

For Western blot analysis, at least four pups per group (ethanol-unexposed, ethanol-unexposed plus resveratrol, ethanol-exposed, and ethanol-exposed plus resveratrol) were used for the isolation of total cerebellum proteins. Tissue samples were homogenized and incubated for 30 min in 1× radiimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) containing phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (aprotinin and leupeptin). Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Extracted proteins were diluted with 5× Laemmli sample buffer and boiled for 5 min. The supernatants were subjected to electrophoresis and analyzed by Western blot. In brief, after electrophoresis, proteins were transferred on polyvinylidene difluoride membrane at 100 V for 3 h in a cold room. Membrane was blocked with 5% nonfat dry milk/TBST (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) followed by incubation in primary antibodies diluted in 2.5% nonfat dry milk/TBST overnight at 4°C. After washing with TBST, membrane was incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG) diluted in 2.5% nonfat dry milk/TBST for 4 h at room temperature. Signals were detected by chemiluminescence detection kit (Pierce/Thermo Fisher Scientific, Rockford, IL). Primary antibodies used were those for cleaved caspase-3 (Cell Signaling Technology), NQO1 (Abcam Inc., Cambridge, MA), HO1 (Enzo Life Sciences, Plymouth Meeting, PA), caspase-7, caspase-8, SOD-2, and Nrf2 from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation of Nuclear Proteins and Electrophoretic Mobility Shift Assay

Nuclear proteins from cerebellar tissue were extracted as described previously (Kumar et al., 2010). A total of four pups per group (ethanol-unexposed, ethanol-unexposed plus resveratrol, ethanol-exposed, and ethanol-exposed plus resveratrol) were used for the isolation of nuclear proteins from the cerebellum. In brief, cerebella were homogenized in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM sulfonyl fluoride (PMSF) and protease inhibitors (aprotinin and leupeptin). Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Extracted proteins were diluted with 5× Laemmli sample buffer and boiled for 5 min. The supernatants were subjected to electrophoresis and analyzed by Western blot. In brief, after electrophoresis, proteins were transferred on polyvinylidene difluoride membrane at 100 V for 3 h in a cold room. Membrane was blocked with 5% nonfat dry milk/TBST (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) followed by incubation in primary antibodies diluted in 2.5% nonfat dry milk/TBST overnight at 4°C. After washing with TBST, membrane was incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG) diluted in 2.5% nonfat dry milk/TBST for 4 h at room temperature. Signals were detected by chemiluminescence detection kit (Pierce/Thermo Fisher Scientific, Rockford, IL). Primary antibodies used were those for cleaved caspase-3 (Cell Signaling Technology), NQO1 (Abcam Inc., Cambridge, MA), HO1 (Enzo Life Sciences, Plymouth Meeting, PA), caspase-7, caspase-8, SOD-2, and Nrf2 from Santa Cruz Biotechnology (Santa Cruz, CA).
PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM NaF, and 0.5 mM Na$_2$VO$_4$ followed by incubation for 10 min on ice. IGEPAL CA-630 (Sigma-Aldrich) was added to a final concentration of 0.5%, and the cell lysate was centrifuged at 3,000g for 10 min. The supernatant (cytoplasmic protein fraction) was collected, and the nuclei pellet was washed once with hypotonic buffer. The pelleted nuclei were incubated with high-salt buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 25% glycerol, 1.5 mM MgCl$_2$, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM NaF, and 0.5 mM Na$_2$VO$_4$) for 30 min on ice. Nuclei were then centrifuged at 12,000g for 15 min. The extracted nuclear protein fraction was dialyzed to remove excess salt and stored at −80°C. Protein concentration was determined by the bicinechonic acid method (Thermo Fisher Scientific).

For electrophoretic mobility shift assay (EMSA), rat NQO1 antioxidant response element (ARE) (5′-TCT AGA GTC ACA GTG ACT-3′) was used as the Nrf2 binding site (Favreau and Pickett, 1991). Double-stranded oligonucleotide was labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) and incubated with 10 μg of nuclear protein extract in binding buffer (10 mM HEPES, pH 7.9, 10 mM MgCl$_2$, 0.02% IGEPAL, 0.5 mM DTT, 50 mM NaCl, and 2 μg of poly(dI-dC), and 10% glycerol) at 25°C for 30 min. Binding reactions were resolved on 6% native polyacrylamide gels containing 0.5 × Tris borate-EDTA buffer (45 mM Tris base, 45 mM boric acid, 8.1% SDS, and 0.8% thiobarbituric acid). The reaction was allowed to proceed for 15 min before labeled probe was added. For supershift assays, 1 μg of the anti-Nrf2 antibody was added in binding reaction 30 min before addition of labeled probe.

**MTT Assay**

To determine the viability of CGNs isolated from cerebellum treated with or without ethanol and/or resveratrol (three pups per group), MTT assay was carried out according to the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). CGNs were incubated with MTT reagent for 4 h and then overnight in solubilization buffer at 37°C. Absorbance of the formazan product was read at 570 nm. A reference wavelength of 690 nm was used to detect background.

**TUNEL Staining**

TUNEL staining for paraffin-embedded brain sections were performed using the DeadEnd fluorometric TUNEL kit (Promega, Madison, WI). Brain sections (6 μm thick) were deparaffinized in xylene, rehydrated with ethanol, and washed with 0.85% NaCl. Tissue sections were fixed with 4% methanol-free formaldehyde solution. After PBS washing, sections were permeabilized with proteinase K solution (20 μg/ml) for 10 min at room temperature. The nicked DNA was labeled with fluorescence-labeled dUTP nucleotide and recombinant terminal deoxynucleotidyl transferase enzyme mix for 60 min at 37°C. After washing with 2× standard saline citrate and PBS, slides were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA) and examined under fluorescence microscopy (Nikon E600; Nokon, Tokyo, Japan).

**Detection of Reactive Oxygen Species**

The production of reactive oxygen species (ROS) in CGNs from ethanol-unexposed, ethanol-exposed, and ethanol-exposed plus resveratrol-treated groups were cultured in Lab-Tek chamber slides (Thermo Fisher Scientific). After 45 min, cells were washed with Hank's buffered salt solution without phenol red and incubated with fluorescence probe carbonyl-H$_2$DCFDA for 30 min at 37°C in the dark. DAPI was added to counterstain the nuclei. Cells were washed three times with HBSS, mounted, and immediately examined (fluorescein isothiocyanate filter) under Nikon E600 fluorescence microscopy. Oxidation of H$_2$DCFDA occurs almost exclusively in the cytosol, and generates a fluorescence that is proportional to ROS generation in that cell. CGNs without dye were processed in parallel as a negative control to detect autofluorescence. As a second negative control, we also monitored fluorescence in a cell-free system in presence of ethanol, horseradish peroxidase (HRP), and carbonyl-H$_2$DCFDA dye at excitation and emission wavelengths of 490 and 520 nm, respectively, for photodynamic and/or atmospheric-oxidation (Myhre et al., 2009).

**Immunohistology**

To determine the alteration in protein expression profile in cerebellum, immunohistochemistry was performed (Kumar et al., 2010). At least four pups per group (ethanol-unexposed, ethanol-exposed, and ethanol-exposed plus resveratrol) were used for immunohistochemical analysis. To detect apoptosis, cleaved caspase-3 antibody (1:500 dilutions; Cell Signaling Technology) was used as primary antibody. For trans-4-hydroxy-2-nonenal (4-HNE) staining, monoclonal anti-HNE antibody (1:250 dilution; Abcam Inc.) was used to detect adduct in tissue samples. Normal IgG (Santa Cruz Biotechnology) was used in place of primary antibody to assess nonspecific staining.

**Oxidative Stress and Antioxidant Enzyme Activity Assay**

For enzyme activity determination, four pups per group (ethanol-unexposed, ethanol-unexposed plus resveratrol, ethanol-exposed, and ethanol-exposed plus resveratrol) were used. After resveratrol and ethanol treatments, cerebella from pups were rapidly excised, thoroughly washed with ice-cold PBS to remove residual red blood cells, and homogenized in buffer containing 0.1 M Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.05% Triton X-100, and 0.5 mM PMSF. After centrifugation at 13,000 rpm at 4°C, supernatant was collected; protein was estimated by BCA kit (Thermo Fisher Scientific) and immediately used for biochemical assays. All the spectrophotometric readings were taken in a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA).

**GSH Level.** The glutathione level in tissue lysate was estimated by luminance based GSH-Glo glutathione kit (Promega, Madison, WI) according to the manufacturer’s instructions. The luminescence was measured in a luminometer (Promega Biosystems, Sunnyvale, CA). The signal generated is proportional to the amount of glutathione present in the sample.

**Total Thiol.** Total thiol in tissue lysate was estimated using 5,5′-dithiobis (2-nitrobenzoic acid) reagent as described by Guleria et al. (2006). In brief, tissue homogenate was mixed with 0.6 ml of 0.25 M Tris and 20 mM EDTA buffer, pH 8.2, followed by addition of 40 μl of 10 mM 5,5′-dithiobis (2-nitrobenzoic acid) and 3.16 ml of absolute methanol. After a 20-min incubation, reaction mixture was centrifuged at 3000g for 10 min. The absorbance of the supernatant was measured at 412 nm. Total thiol in the sample was expressed in millimol per milligram of protein in the lysate with a molar extinction coefficient of 13,600 M/cm for calculation.

**Lipid Peroxidation.** The antilipid peroxidation effect of resveratrol in ethanol-treated cerebellum was determined by the formation of thiobarbituric acid reactive substances and expressed as the extent of malondialdehyde (MDA) produced as a result of lipid peroxidation (Guleria et al., 2006). The tissue lysate was mixed with 20% acetic acid, 8.1% SDS, and 0.8% thiobarbituric acid. The reaction mixture was placed in a boiling water bath for 1 h. After cooling under tap water, reaction mixture was centrifuged at 3000g for 5 min, and the absorbance of the supernatant was read at 532 nm. The molecular extinction coefficient of 1.56 × 10$^5$ M/cm is used to calcu-
late nanomoles of MDA formed per milligram of protein in the lysates.

**Superoxide Dismutase Activity.** Superoxide dismutase (SOD) activity in tissue samples was determined using SOD determination kit (Sigma-Aldrich) according to the manufacturer's instructions. This assay uses a water-soluble tetrazolium, the sodium salt of 4-[3(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, to detect superoxide radical generated by xanthine oxidase in the presence of superoxide dismutase.

**8-iso-PGF2α Assay.** Levels of the 8-epimer of prostaglandin F2α (8-iso-PGF2α) reflecting the lipid peroxidation were estimated using a kit according to manufacturer's instructions (direct 8-iso-PGF2α enzyme immunoassay kit (Enzo Life Sciences). In brief, after decapitation, cerebellar tissue from resveratrol-fed ethanol-treated or untreated pups was quickly stored in liquid nitrogen until use. Tissue samples were powdered in liquid nitrogen, hydrolyzed with 1 ml of 2 N NaOH at 45°C for 2 h, followed by neutralization with an equal volume (1 ml) of 2 N HCl. After centrifugation at 3000 rpm, clear supernatant was used to estimate 8-iso-PGF2α using enzyme-linked immunosorbent assay-based Direct 8-iso-PGF2α enzyme immunoassay kit. The absorbance of developed yellow color was read at 405 nm in a spectrophotometer. The standards in the range of 160 to 10² pg/ml were also run in parallel.

**Data Analysis**

Data are presented as the mean and S.D. Comparisons were made among the groups using the one-way analysis of variance test followed by Tukey-Kramer ad hoc test (GraphPad software, La Jolla, CA). A p value < 0.05 was considered significant.

**Results**

**Resveratrol Prevents Ethanol-Induced Apoptosis in the Cerebellum.** Our previous studies have shown that 80

![Fig. 1. A, Western blot analysis to determine the effect of different concentrations of resveratrol (2, 20, 40, or 100 mg/kg) on ethanol-induced activation of caspases in the cerebellum. Whole-cell extracts from the cerebellum of ethanol-exposed or unexposed and/or resveratrol (Res)-fed pups were prepared using radioimmunoprecipitation assay lysis buffer. Equal amounts of proteins were used for Western blotting using anti-cleaved caspase-3, -7, and -8 antibodies. β-Actin was used to check the loading difference. Bands were quantified by densitometric scanning and normalized to the level of β-actin. Data represent average fold change relative of control (ethanol-unexposed pups) from at least three independent experiments (mean ± S.D.). ns, nonsignificant compared with ethanol-unexposed pups. B, immunofluorescence staining for cleaved caspase-3 in the cerebellum. Resveratrol alone had no effect on the caspase-3 activation in ethanol-unexposed control group (c–j). Ethanol induces activation of caspase-3 (k), whereas resveratrol at 100 mg/kg reduced the activation of caspase-3 in the EGL (indicated by arrow) of ethanol-exposed cerebellum (s). DAPI was used to stain nuclei; images were overlapped to monitor the boundary of cerebellum. The enlarged images u and v show cleaved caspase-3 staining of EGL of ethanol-exposed cerebellum. Scale bar, 50 μm. Pictures are representative of at least three independent experiments.**
mM ethanol induces apoptosis in the external granule layer of cerebellum in PD7 pups (Joshi et al., 2006). To further test whether resveratrol prevents apoptosis by preventing the activation of effector caspases, we isolated total proteins from the cerebellum of ethanol-exposed or unexposed and/or resveratrol-fed pups and performed Western blot analysis. After activation, caspase-3 is cleaved to yield a smaller active fragment (19 kDa). The presence of a 19-kDa band in our Western blots of extracted proteins isolated from cerebellum of ethanol-exposed pups indicated the activation of caspase-3 (Fig. 1A). The intensity of the active band (19 kDa) remained almost unchanged when 2, 20, or 40 mg/kg resveratrol was administered before ethanol exposure. However, it was significantly reduced (~3.5-fold compared with ethanol-exposed pups) when higher doses of resveratrol (100 mg/kg) were used under similar conditions (Fig. 1A). Neither dose of resveratrol affected the intensity of caspase-7 and caspase-8 bands in protein samples isolated from the cerebellum of unexposed and ethanol-exposed pups (Fig. 1A).

To detect the generation of cleaved active caspase-3 in the cerebellum, we also performed immunohistochemistry with a specific antibody against cleaved caspase-3. In ethanol plus resveratrol (100 mg/kg)-treated pups, the intensity of cleaved caspase-3 staining in the external granule layer (EGL) of cerebellum was significantly lower than that of ethanol-exposed pups (Fig. 1B, k and s). However, the fluorescence intensity for cleaved caspase-3 in the cerebellum of ethanol-exposed pups administered lower doses of resveratrol (2, 20, or 40 mg/kg) was unaffected (Fig. 1B).

To determine whether resveratrol protects against ethanol-induced apoptosis in cerebellum, we performed TUNEL assays to detect DNA fragmentation. Ethanol exposure for 5 h induced apoptosis in the external granule layer of the cerebellum as indicated by the TUNEL-positive cells (Fig. 1C, k). The staining with DAPI, as well as fragmented DNA (TUNEL-positive staining) in ethanol-exposed cerebellum, overlapped in a single cell (shown in enlarged image), suggesting that ethanol promotes DNA fragmentation and apoptosis in cerebellum (Fig. 1C, u–w). After treatment with 100 mg/kg resveratrol, the number of TUNEL-positive cells in the cerebellum were markedly reduced with ethanol exposure. Resveratrol alone had no effect on DNA fragmentation, as shown in Fig. 1C, c to j, and low doses of resveratrol (2, 20, or 40 mg/kg) did not significantly reduce the number of TUNEL-positive cells in response to 80 mM ethanol.

**Resveratrol Prevents Ethanol-Induced Production of ROS in the Cerebellum and in Cerebellar Granule Cells.** Ethanol increases oxidation of lipids in the cerebellum that results in the formation of reactive aldehyde adduct 4-HNE. By staining tissue samples with a specific antibody against HNE, we demonstrated increased lipid peroxidation in the external granule layer of cerebellum of ethanol-treated samples (Fig. 2A, k). Administration of 40 or 100 mg/kg (but not 2 or 20 mg/kg) resveratrol reduced the formation of HNE adduct in the cerebellum of ethanol-exposed pups (Fig. 2A, q and s). These data indicate that 40 or 100 mg/kg resveratrol prevents the ethanol-induced increase in lipid peroxidation.

To test whether resveratrol could suppress ROS formation, we performed H$_2$DCFDA staining in freshly isolated CGNs and counted the cells containing the dye (Karlsson et al., 2010). The assay, based on H$_2$DCFDA oxidation by ROS and removal of the acetate group by cellular esterases to the fluorescent molecule CM-H$_2$DCFDA, has been used frequently to measure oxidative stress, and representative staining is shown in Fig. 2B. In the present study, ~80% of CGNs isolated from ethanol-exposed cerebellum stained positive for H$_2$DCFDA compared with control (p < 0.01 compared with ethanol-unexposed). The percentage of stained CGNs from the ethanol plus 40 or 100 mg/kg resveratrol group was significantly reduced by ~30 or ~60%, respec-

![Fig. 1. Continued.](image-url)
tively \((p < 0.05\) compared with ethanol-exposed; Fig. 2C), indicating an antioxidant effect of resveratrol. There was no staining when cells were processed without \(H_2\text{DCFDA}\) dye (data not shown). As an additional control, we used a cell-free system with buffer containing dye, horseradish peroxidase, and ethanol (Myhre et al., 2003). As shown in Fig. 2D, ethanol alone was not able to oxidize \(H_2\text{DCFDA}\) to the fluorescent compound carboxyl-dichlorofluorescein. After addition of horseradish peroxidase (5 units/ml) in the mixture of buffer, dye (25 \(\mu\)M), and ethanol (80 mM), the OD value increased by 4% after 15 sec compared with dye plus HRP alone. It increased by \(-13\%\) after 6 min, indicating the increased oxidation of \(H_2\text{DCFDA}\) to the fluorescent compound carboxyl-dichlorofluorescein. The OD of only HRP plus buffer reaction.

**Fig. 2.** A, determination of oxidative stress. Immunofluorescence staining to determine the effect of resveratrol on ethanol-induced formation of 4-HNE protein adduct in the cerebellum. Nuclei were counterstained with DAPI; images were overlapped to monitor the boundary of cerebellum, and slides were examined under a stereo microscope (Olympus, Center Valley, PA). No significant staining was observed in control groups (a, c, e, g, and i). Ethanol increased lipid peroxidation in EGL as shown by HNE staining (k), and resveratrol (40 and 100 mg/kg) prevented increased HNE staining (q and s). Scale bar, 1 mm. Pictures are representative of at least three independent experiments. \(H_2\text{DCFDA}\) staining was performed to measure the ROS in CGNs (B). CGNs harvested from untreated and resveratrol-treated groups were cultured in chamber slides and stained with \(H_2\text{DCFDA}\) dye as described under Materials and Methods (a–f). Nuclei were counterstained with DAPI (g–l). Oxidation of \(H_2\text{DCFDA}\) occurs almost exclusively in the cytosol, generating fluorescence that is proportional to ROS generation. Ethanol exposure increased the generation of ROS, indicated by the increased intensity of \(H_2\text{DCFDA}\) dye in CGNs, as shown by arrows (B, b). An enlarged image of a single CGN stained with DAPI and \(H_2\text{DCFDA}\) dye is shown in B, h. Reduced intensity of \(H_2\text{DCFDA}\) in CGNs in B, f and l, indicates that resveratrol prevented generation of ROS. C, bar diagram shows the percentage of CGNs with \(H_2\text{DCFDA}\) staining counted from treated and untreated groups. Pictures are representative of at least three independent experiments. Scale bar, 50 \(\mu\)m. Bars are mean and S.D. of three independent experiments.
was increased in the first 15 s and then remained constant. These data indicate that nonfluorescent H$_2$DCFDA dye was oxidized into the fluorescent compound by ethanol in combination with cellular peroxidases.

**Resveratrol Maintains High Cell Viability in Ethanol-Exposed Cerebellar Granule Cells.** To evaluate a potential effect of resveratrol on cell viability, we also examined the morphology of CGNs under light microscope (phase contrast as dead cells and phase bright as live cells) and survival of CGNs by MTT assay. For studying cell morphology, CGNs were isolated from resveratrol-fed pups, both ethanol-treated and -untreated, and cultured for 2 days to assess the differentiation. In ethanol-untreated CGNs, cell viability did not differ notably among different groups, as evaluated by number of phase contrast versus phase bright cells (Fig. 3A). The proportion of live cells isolated from untreated and 2, 20, 40, or 100 mg/kg resveratrol-fed pups were 94.8,
95, 96, 95.7, and 95.3%, respectively. Ethanol exposure reduced the percentage of live CGNs (17.2%); however, 18, 20, 59, and 86.9% of CGNs remains viable in 2, 20, 40, and 100 mg/kg resveratrol fed ethanol-exposed pups, respectively (Fig. 3B). To further confirm the effect of resveratrol on viability of CGNs, we performed MTT assay (Fig. 3C). Similar OD values for cell viability were found among control groups (ethanol-unexposed, 2.9; resveratrol-fed 2 mg/kg, 2.8; 20 mg/kg, 2.8; 40 mg/kg, 2.6; and 100 mg/kg, 2.3) \( (p > 0.05) \). The OD values were significantly decreased in ethanol-exposed CGNs (OD 1.1; \( p < 0.05 \) compared with ethanol-unexposed pups) indicative of lower cell viability. Resveratrol (100 mg/kg) administration prevented the reduction in cell viability resulting from ethanol exposure in CGNs (OD 2.5; \( p < 0.05 \) compared with ethanol-exposed pups). Unlike the previous finding on morphological evaluation by light microscopy (Fig. 3B), resveratrol at 40 mg/kg did not prevent ethanol effects on survival of CGNs by MTT assay (Fig. 3C).

### Resveratrol Prevents Ethanol-Induced Oxidative Stress in the Cerebellum.

The reduced form of glutathione (GSH) and GSH-related enzymes are involved in the detoxification of \( \text{H}_2\text{O}_2 \) and lipid peroxidation and have been suggested to play important roles in protecting against oxidative stress. We therefore tested the effect of resveratrol on the total GSH levels in cerebellum. Ethanol exposure reduced total GSH levels in the cerebellum (\( p < 0.01 \) compared with ethanol-exposed pups), and this ethanol-induced GSH reduction was prevented when resveratrol was administered at 100 mg/kg (\( p < 0.01 \) compared with ethanol-exposed pups; Fig. 4A). The elevation in GSH level was not statistically significant when resveratrol 2, 20, or 40 mg/kg was administered to ethanol-exposed pups (Fig. 4A). Ethanol exposure significantly elevated total thiol levels in the cerebellum (\( p < 0.001 \) compared with ethanol-unexposed pups). Administration of resveratrol significantly blocked the ethanol-induced increase (for 40 and 100 mg/kg, \( p < 0.05 \) and \( p < 0.001 \), respectively, compared with the ethanol-exposed group) in total thiol levels in the cerebellum (Fig. 4B).

No significant differences in cerebellar levels of MDA, an indicator of lipid peroxidation, were observed in resveratrol and the control group. Ethanol exposure significantly increased (\( p < 0.05 \) compared with ethanol-unexposed) lipid peroxidation in the cerebellum, as indicated by increased levels of MDA (Fig. 4C). Resveratrol markedly suppressed (for 40 and 100 mg/kg, \( p = 0.05 \) and \( p < 0.05 \), respectively, compared with ethanol-exposed group) ethanol-induced MDA generation (Fig. 4C). We also measured the level of 8-iso-PGF2\( \alpha \) in the cerebellum in response to ethanol exposure. The 8-iso-PGF2\( \alpha \), a stable P2 isoprostane, is produced in vivo by the nonenzymatic peroxidation of arachidonic acid and has been considered a reliable oxidative stress marker to detect lipid peroxidation by free radicals in tissues (Morrow et al., 1990; Gopaul et al., 2000). In our experimental conditions, 80 mM ethanol increased the level of 8-iso-PGF2\( \alpha \) generation in cerebellum by \( \sim 2.5 \)-fold compared with ethanol-ununtreated pups. Although resveratrol (40 mg/kg) reduced the levels of 8-iso-PGF2\( \alpha \) in the cerebellum of ethanol-exposed pups, a higher dose of resveratrol (100 mg/kg) was more effective (\( p < 0.001 \); Fig. 4D). The altered levels of lipid peroxidation, GSH, and total thiol in response to ethanol exposure were not significantly altered by administration of 2 or 20 mg/kg resveratrol (\( p > 0.05 \) versus ethanol-exposed pups).

### Resveratrol Maintains the DNA-Binding Activity of Nrf2 upon Ethanol Exposure.

The nuclear factor-erythroid derived 2-like 2 (nfe2l2, also known as Nrf2) acts as a transcription factor and binds to the ARE of genes that protect against oxidative damage. To determine whether resveratrol affects the nuclear distribution of Nrf2 in cerebellum, we isolated nuclear proteins from the cerebellum of different treatment groups of pups for Western blotting using an anti-Nrf2 antibody. Neither the total nor the cytoplasmic level of Nrf2 changed in response to ethanol and resveratrol (\( p > 0.05 \) compared with ethanol-unexposed pups; Fig. 5, A and B, a). However, as shown in Fig. 5B, b, the level of nuclear Nrf2 was decreased significantly in ethanol-exposed cerebellum (\( p < 0.001 \) compared with ethanol-unexposed pups), whereas administration of resveratrol (100 mg/kg) in ethanol-exposed pups prevented the loss of nuclear Nrf2 protein level (\( p < 0.001 \) compared with ethanol-exposed pups) and maintained Nrf2 levels near that of ethanol-unexposed cerebellum. Resveratrol less than 100 mg/kg (i.e., 2, 20, or 40 mg/kg) did not increase the level of Nrf2 in nuclear fraction of ethanol-exposed cerebellar tissue. Similar results were obtained with nuclear proteins isolated from the CGNs prepared from different treatment groups of pups (Fig. 5B, b).

To determine whether ethanol-induced changes in nuclear abundance of Nrf2 also affect expression of Nrf2-regulated proteins (such as NQO1, HO1, and SOD), we performed Western blot analysis to determine their expression and also performed an activity assay for SOD. In our Western blots, the protein level of NQO1 and SOD2 was decreased in ethanol-treated cerebellum (\( p < 0.01 \) compared with ethanol-unexposed pups), although it was restored by 100 mg/kg resveratrol in ethanol-exposed pups (Fig. 5, C and D). Administration of 40 mg/kg resveratrol in ethanol-fed pups significantly increased SOD2 protein level (\( p < 0.01 \)) but not that of NQO1 protein in cerebellum (\( p > 0.05 \)). Neither 2 nor 20 mg/kg resveratrol restored the protein level of NQO1 or SOD2 in cerebellum of ethanol-exposed pups (Fig. 5, C and D). The expression level for HO1 protein remains unchanged in different treatment groups (\( p > 0.05 \)) compared with ethanol-unexposed pups; Fig. 5, C and D). The activity of SOD was significantly decreased in ethanol-exposed pups compared with untreated groups (\( p < 0.001 \) compared with ethanol-unexposed pups), and administration of 40 or 100 mg/kg resveratrol resulted in significant elevation of SOD activity (for 40 and 100 mg/kg, \( p < 0.05 \) and \( p < 0.001 \), respectively, compared with ethanol-exposed group) in ethanol-exposed pups (Fig. 5G). We also measured the DNA-binding activity of Nrf2 by EMSA. Nuclear extracts prepared from cerebella of ethanol-exposed pups exhibit decreased DNA-binding activity of Nrf2 to an oligonucleotide harboring the rat NQO1 ARE compared with nuclear extract prepared from ethanol-untreated cerebellum (Fig. 5E, lanes 4 and 16). The intensity of protein-DNA complexes was maintained near control levels in nuclear proteins isolated from ethanol-exposed cerebellum with 100 mg/kg resveratrol administration (Fig. 5E, lanes 5 and 20). The binding was specific, as addition of excess unlabeled oligonucleotide competed off the binding (Fig. 5E, lane 6). Inhibition of DNA-protein complexes by addition of Nrf2 antibody (1 \( \mu \)g) in EMSA reaction further confirms the specificity of Nrf2 binding with the rat NQO1.
Fig. 5. Resveratrol prevents ethanol-induced loss of Nrf2 level and its DNA-binding activity in the nucleus. Total cerebellar proteins were isolated and separated by SDS-polyacrylamide gel electrophoresis, and Western blot was performed with anti-Nrf2 antibody. Blot was probed to β-actin to check the loading differences (A, top). Protein bands were quantified by densitometric scanning and normalized to the level of β-actin. Data represent average fold increase relative to control (ethanol-unexposed pups) from at least three independent experiments (mean ± S.D.). ns, nonsignificant compared with ethanol-unexposed pups (A, bottom). Determination of Nrf2 level in cerebellar proteins isolated from cytoplasmic and nuclear fractions (B). Equal amounts of cytoplasmic (B, a) and nuclear (B, b) proteins of cerebellar tissue (top) and CGN (middle) isolated from ethanol and/or resveratrol-fed pups were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting using antibodies for Nrf2 (top) and β-actin or transcription factor IID to assess loading (bottom). Protein bands were quantified by densitometric scanning and normalized to the level of β-actin or TFIID. Data represent average fold change relative to control (ethanol-unexposed pups) from at least three independent experiments (mean ± S.D.). ns, nonsignificant compared with ethanol-unexposed pups. The level of Nrf2-regulated proteins was assessed by Western blot analysis using total cerebellum proteins and probed with anti-NQO1, HO1, and SOD2 antibodies. Membranes were probed with β-actin as a control for equal loading (C). Bands were quantified by densitometric scanning and normalized to the level of β-actin. Data represent average fold change relative of control (ethanol-unexposed pups) from at least three independent experiments (mean ± S.D.). ns, nonsignificant compared with ethanol-unexposed pups (D). EMSA was carried out to determine the DNA-binding activity of Nrf2. Nuclear protein (10 μg) isolated from cerebellum (E) and from CGNs...
Discussion

Ethanol is one of the most potent neurotoxic substances, and a high dose of ethanol during embryonic development causes dysfunction of the central nervous system (Olney et al., 2000). Previous reports have demonstrated that apoptosis is the major factor responsible for ethanol-induced neuronal cell death in the cerebellum, which may occur via the activation of caspase-3 as shown in our studies (Fig. 1A). The protein expression level of caspase-7 and -8 did not differ significantly in any group, suggesting that caspase-7 and -8 are not involved in ethanol-induced apoptosis in the cerebellum. Our findings are consistent with previous reports showing that activation of caspase-3 is responsible for ethanol-induced neuronal cell death in the developing brain (Olney et al., 2002). Activated caspase-3 has been shown to induce multiple cellular events that trigger a range of downstream apoptotic events, such as cell shrinkage, chromatin condensation, membrane blebbing, and DNA fragmentation (Nagata et al., 2003).

Resveratrol has been shown to be a promising natural compound containing antiapoptotic, free radical-scavenging, and antilipoprotein peroxidation properties (Shakibaei et al., 2009). To test whether resveratrol has an antiapoptotic effect against ethanol in cerebellum, ethanol-exposed pups were fed with different doses of resveratrol (2, 20, 40, or 100 mg/kg). Figure 1A clearly indicates that resveratrol administration at 100 mg/kg significantly prevented ethanol-induced activation of caspase-3 in the cerebellum, whereas the lower concentrations of resveratrol (20, 40 mg/kg) had no effect on caspase-3 activation. The reduced level of cleaved caspase-3 in ethanol-exposed cerebellum by resveratrol suggests that the neuroprotective effect of resveratrol may result from reduced neuronal apoptosis in cerebellum. The increased level of activated caspase-3 triggers DNA fragmentation in ethanol-exposed cerebellum (as more TUNEL-positive cells in ethanol-exposed cerebellum than control groups); however, resveratrol (100 mg/kg; not lower concentrations) administration dramatically reduced DNA fragmentation in ethanol-exposed cerebellum (Fig. 1C).

Using H2DCFDA staining, we further demonstrated that ethanol-induced apoptosis resulted from the generation of ROS in CGNs (Fig. 2B). However, Heaton et al. (2002) detected no increase in ROS level 2 h after ethanol exposure whereas ROS level was significantly decreased at 12 or 24 h after ethanol exposure in whole cerebella of postnatal day 7 pups. These differences in the results might be due to the analysis of ROS level in CGNs and different duration of ethanol exposure in our experiments. ROS, such as free radicals and peroxides, are the major cause of cellular oxidative stress, which oxidizes the biomolecules (such as oxidation of thiol groups, lipid peroxidation, and oxidation of bases in nucleic acids). All these events lead to harmful effects, such as generation of highly reactive aldehyde byproducts, protein aggregation, and degradation (Kannan and Jain, 2000). ROS generated by several metabolic pathways are detoxified by the SOD to prevent apoptosis in neurons (Greenlund et al., 1995). The results in the present study demonstrate that 40 and 100 mg/kg resveratrol restored protein expression (Fig. 5C) as well as enzymatic activity of SOD (Fig. 5G), decreased the level of total thiol, and reduced the oxidation of lipids in ethanol-exposed cerebellum (Fig. 4). In addition, treatment with resveratrol prevented ethanol-induced depletion of intracellular GSH level in the cerebellum (Fig. 4A). All these events indicated that antioxidative property of resveratrol is responsible for prevention of ethanol-induced oxidative stress in CGNs (Fig. 2B). The MTT assay, as well as morphological studies (by light microscopy), further revealed the protective role of resveratrol in the survival of ethanol-exposed cerebellar neurons. In our experimental conditions, lower doses of resveratrol (2 or 20 mg/kg) were not able to restore the levels of GSH, total thiol, or oxidation of lipids. Slightly higher dose of resveratrol (40 mg/kg) was partially effective in preventing oxidative stress in the cerebellum. However, these effects were insufficient in preventing apoptosis and improving the viability of CGNs (Fig. 3, A and C). High doses of resveratrol prevented ethanol effects on survival of CGNs, indicating that it was able to overcome the effects of ethanol on oxidative stress.

It is well documented that ethanol metabolism results in the formation of electrophilic aldehydes (4-hydroxynonenal and 4-oxo-2-nonenal) as byproducts of peroxidation of lipid membrane (Niemelä, 1999). 4-HNE exerts various biological effects in various cell types, such as alterations in cell proliferation and apoptosis (Ong et al., 2000). There is also evidence that HNE induces apoptosis in cultured cerebellar granule cells (Ito et al., 1999), in primary rat hippocampal neurons and PC12 cells (Kruman et al., 1997), and in fetal brain (Ramachandran et al., 2001). The toxic HNE has been shown to be detoxified by the conjugation with GSH, protecting PC12 and hippocampal neurons from apoptosis induced by oxidative stress as well as by HNE (Kruman et al., 1997). Our studies show that ethanol metabolism increases oxidative stress, which enhances lipid peroxidation as well as the level of HNE in cerebellum (Fig. 2A). The elevated level of GSH (compared with the ethanol-treated group) by administration of resveratrol (100 mg/kg) might detoxify the increased level of HNE and protects the neurons from ethanol-induced cell death.

To delineate the protective mechanism exerted by resveratrol against ethanol-induced toxicity, we focused on Nrf2-mediated signaling. Growing evidence supports a role of Nrf2 signaling in protecting cells from oxidative insults. Under
normal conditions, Nrf2 remains associated with a Kelch-like ECH-associated protein1 (Keap-1) in cytoplasm (Kobayashi and Yamamoto, 2005). Nrf2 inducers such as antioxidants or electrophilic compounds dissociate the Nrf2/Keap1 complex by modifying the thiol group of Nrf2 or Keap1 and/or phosphorylation by protein kinase C; as a result, activated Nrf2 is transported to the nucleus, where it is oligomerized with small proteins and interacts with AREs present upstream of several detoxifying enzymes (such as catalase, superoxide dismutase, UDP-glucuronosyltransferase, NADPH quinine oxidoreductase 1, heme oxygenase 1, glutathione peroxidase, and γ-glutamylcysteine synthetase) and modulates their expression (Jaiswal, 2000). These enzymes are involved in glutathione and NADPH production as well as in maintaining intracellular redox homeostasis.

To address the differential regulation of detoxifying and antioxidant enzymes in response to ethanol and resveratrol, we assessed the nuclear level as well as the DNA-binding activity of Nrf2. Our studies indicate that ethanol reduces nuclear abundance of Nrf2 as well as DNA binding activity of Nrf2 to ARE sequence of NQO1 compared with ethanol-untreated pups, whereas resveratrol prevents the loss of nuclear Nrf2 as well as its DNA binding activity in ethanol-treated cerebellum (Fig. 5, B and E) as well as in CGNs (Fig. 5, B and F). Contrary to previous observation on the transient Nrf2 accumulation in nuclei after oxidative insult (Jain and Jaiswal, 2007), we found ethanol-induced reduction in the levels of Nrf2 in the nuclei. It might be due to high-dose ethanol and use of CGNs in our studies. In a related study (Rogo et al., 2008), it was shown that direct activation of glycogen synthase kinase 3β results in nuclear exclusion of Nrf2 in CGNs. This unusual response to oxidative stress might be responsible for loss of tolerance to persistent oxidant exposure, as found in various neuropathological conditions (Rojo et al., 2008). Because ethanol activates glycogen synthase kinase 3β in developing cerebellum and in CGNs (Luo, 2009), it might lead to reduction in Nrf2 levels in the nuclei.

Western blot analysis of Nrf2-regulated downstream proteins (herein NQO1 and SOD) indicates that the ethanol exposure decreases the level of these proteins in cerebellum, whereas it was restored in the cerebella of ethanol-exposed 100 mg/kg resveratrol-fed pups (Fig. 5C). In contrast to NQO1 and SOD expression, the cerebellar protein level of HO1 remains unchanged in response to ethanol in our experimental conditions. HO1 enzyme activity, highly expressed in spleen and liver, is involved in heme biosynthesis and catalyzes intracellular heme into bilirubin. However, in guinea pig cerebellum, ethanol exposure (in vitro, short- and long-term in vivo) has no effect on HO1 activity (Cook et al., 1997).

Yeligar et al. (2010) has reported that ethanol regulates HO1 and NQO1 transcription by different signaling pathways in Kupffer cells. Thus, besides Nrf2, other protein factor(s) interacting with regulatory elements might be involved in regulation of HO1 in response to ethanol-induced oxidative stress in the cerebellum.

These results lead us to hypothesize that reduced levels of Nrf2 in the nucleus resulting from ethanol exposure alone decreases its binding to AREs, which would in turn be expected to lower the expression of antioxidant enzymes, as we found with NQO1 and SOD proteins (Fig. 5C). The lower level of antioxidant enzymes would fail to remove the reactive oxygen species and lead to neuronal cell death by ethanol. Resveratrol administration to ethanol-exposed pups restores the Nrf2 protein level in the nucleus to almost similar levels present in cerebellar nuclear fraction of ethanol-untreated pups. The ability of resveratrol to preserve Nrf2-DNA binding activity in ethanol-treated cerebellum as well as in CGNs (as shown in EMSA results; Fig. 5, E and F) may maintain sufficient transcriptional activation of various antioxidant genes to balance the redox homeostasis in neurons, thereby promoting cell survival under ethanol-exposed conditions.

Acknowledgments

We thank members of the SOM instrumentation resource facility (IRF) for their assistance in fluorescence microscopy and histology.

Authorship Contributions

Participated in research design: Kumar and U. S. Singh.

Conducted experiments: Kumar.

Contributed new reagents or analytic tools: C. K. Singh and LaVoie.

Performed data analysis: Kumar, LaVoie, DiPette, and U. S. Singh.

Wrote or contributed to the writing of the manuscript: Kumar, LaVoie, DiPette, and U. S. Singh.

References


Address correspondence to: Dr. Ugra S. Singh, Department of Pathology, Microbiology, and Immunology, School of Medicine, University of South Carolina, 6311 Garners Ferry Road, Columbia, SC 29209. E-mail: ugra.singh@uscmed.sc.edu