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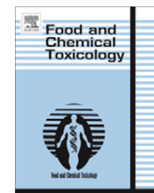

Neuroprotective effects of resveratrol on embryonic dorsal root ganglion neurons with neurotoxicity induced by ethanol

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ABSTRACT

Studies have established that ethanol (EtOH) consumption results in damage to the peripheral nervous systems. Although the pathobiological mechanism is still unclear, oxidative stress is known to play an important role in EtOH-induced neurotoxicity. Because resveratrol (Res) is attracting increased attention due to its antioxidative properties, we investigated the neuroprotective efficacy of Res in ethanol-treated embryonic dorsal root ganglion (DRG) neurons *in vitro*. Organotypic DRG explants and a dispersed cell culture model were used to evaluate the effects of Res on EtOH-induced neurotoxicity. Res increased the number of extended nerve fibers and neurons that migrated from the DRG explants. Hoechst 33342 staining and terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling analysis showed that the EtOH-induced apoptosis was inhibited by Res. The effects of Res were blocked by the 5′-adenosine monophosphate-activated protein kinase inhibitor Compound C and the sirtuin 1 inhibitor nicotinamide. The elevation of oxidative/nitrosative stress, as measured by the amount of reactive oxygen species, malondialdehyde, nitrite, glutathione and superoxide dismutase activity, was also attenuated by Res. The data from the present study indicate that Res protects DRG neurons from EtOH-induced neurotoxicity. Res and its derivative may be effective for the treatment of diseases characterized by axonopathy and neuron loss induced by EtOH.

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1. Introduction

Clinical studies and research in animals have established that ethanol (EtOH) consumption results in damage to the central and peripheral nervous systems and causes many diseases associated with neuronal degeneration, such as fetal alcohol spectrum disorder (Farber et al., 2010) and painful peripheral neuropathy (Kandhare et al., 2012). Although EtOH is thought to exert a direct neurotoxic action and induce neuronal death during EtOH intoxication, the underlying mechanisms of the EtOH-induced neuropathological effects remain unclear (Ramachandran et al., 2003; Young et al., 2003). Recently, many studies have demonstrated that EtOH-induced neuronal death is related to increases in oxidative stress that coincide with the induction of oxidative enzymes (Crews and Nixon, 2009). The amelioration of the EtOH-mediated increases in the production of reactive oxygen species (ROS) contributes to the survival of neuronal cells in the neonatal rat cerebellum (Chu et al., 2007; Heaton et al., 2006).

The beneficial effects of antioxidant therapy during EtOH exposure also support the role of oxidative stress in EtOH-induced neurotoxicity. Nuclear factor-erythroid 2-related factor two induces endogenous antioxidants and has been shown to prevent oxidative stress and apoptosis in EtOH-exposed mouse embryonic cranial neural crest cells (Yan et al., 2010). EtOH augments apoptosis in fetal rhombencephalic neurons, and co-treatment with antioxidants, such as alpha-lipoic acid or N-acetyl cysteine, prevents EtOH-associated apoptosis (Antonio et al., 2011). However, whether antioxidants are effective in attenuating the EtOH-induced loss of neurons during development is still unknown (Antonio and Druse, 2008; Edwards et al., 2002).

Abbreviations: AMPK, 5′-adenosine monophosphate (AMP)-activated protein kinase; CC, Compound C; DAPI, 4′,6-diamidino-2-phenylindole; DRG, dorsal root ganglia; E, embryonic day; GSH, glutathione; GSK3β, glycogen synthase kinase 3β; LKB1, serine/threonine kinase 11; MAP2, microtubule-associated protein 2; MDA, malondialdehyde; NCA, nicotinamide; NGF, nerve growth factor; PAR, polyADP-ribose; PARP-1, poly(ADP-ribose) polymerase-1; PBS, phosphate buffered saline; PGC-1α, proliferator-activated receptor-gamma coactivator-1α; Res, resveratrol; RNS, reactive nitrogen species; ROS, reactive oxygen species; SIRT1, sirtuin 1; SOD, superoxide dismutase; WST-1, water soluble tetrazolium salt-1.

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Chronic and excessive EtOH consumption can lead to alcoholic peripheral neuropathy, which is most prevalent in the 40- to 70-year-old age group and is encountered in both males and females in the Western hemisphere (Peters et al., 2006). In male Wistar rats, chronic, heavy ethanol exposure increased the proportion of vacuolated neurons in the sensory inferior vagal ganglion and the dorsal root ganglion (DRG). Some vacuolated neurons showed dilated endoplasmic reticulum and mitochondrial alterations (Jaatinen et al., 1994). EtOH administration inhibits the regeneration of transected and then sutured sciatic nerves, impairing the growth of axons in the transected nerve and destroying the regenerating sensory ganglion cells (Zimnoch et al., 2000). EtOH treatment caused the loss of cells in the neural crest region, which migration leading to DRG formation, in whole embryo culture study and leading to increased apoptosis in embryonic day 14 (E14) DRG neural stem cells (Anthony et al., 2008). An investigation of the effect of chronic ethanol exposure suggested that the cultured adult DRG neurons showed a slightly greater degeneration with increasing ethanol than fetal neurons (Scott et al., 1986). While EtOH-induced injury to DRG neurons is marginally relieved by the available therapies, antioxidants seem to be effective for alleviating EtOH-induced nerve injury (Kandhare et al., 2012).

In this study, we investigated the neuroprotective efficacy of resveratrol (Res) in EtOH treated DRG neurons *in vitro*. We postulated that the administration of Res would restore antioxidant levels and improve neuronal survival in EtOH-treated DRG neurons. Res, a polyphenolic compound found in grapes, red wine, berries, peanuts and other plants, has antioxidative properties (Smoliga et al., 2011). Res is attracting increased attention due to its health benefits in neurological conditions (Marques et al., 2009). Res has been shown to activate AMP-activated protein kinase (AMPK) and promote robust neurite outgrowth in Neuro2a cells (Dasgupta and Milbrandt, 2007). Res also may be effective for the treatment of diseases characterized by axonopathy and neurodegeneration due to its ability to activate sirtuin 1 (SIRT1) (Araki et al., 2004; Wang et al., 2011). Res has been shown to activate the antioxidant response and prevent hyperglycemic oxidative stress in DRG neurons (Vincent et al., 2009). We hypothesized that Res administration in embryonic DRG culture model would improve neuronal survival by suppressing oxidative stress. The present study focused on the effects of Res on neurite outgrowth, neuronal migration, apoptosis and oxidative stress of embryonic DRG neurons with EtOH-induced toxicity *in vitro*.

2. Materials and methods

2.1. Cell isolation and culture

All culture preparations originated from rats taken from the breeding colony of Wistar rats maintained in the Experimental Animal Center at Shandong University of China. One hundred embryonic rats at E15 were used for DRG culture preparations. All procedures described herein were reviewed by and had the prior approval of the Ethical Committee for Animal Experimentation of the Shandong University. All surgery was performed under anesthesia, and all efforts were made to minimize suffering. Under aseptic conditions, the bilateral DRGs were removed from each embryo and digested with 0.25% trypsin (Sigma, USA) and 0.3 mg/mL collagenase type I in D-Hanks solution at 37 °C for 10 min. After dissociation, 10% fetal bovine serum was added to stop digestion, and the cells were centrifuged for 5 min at 1000 rpm. The cells were then resuspended and triturated in Dulbecco's Modified Eagle Medium with the F-12 supplement (DMEM/F-12) (Invitrogen, USA), which consisted of 10% heat inactivated fetal bovine serum (Invitrogen, USA), 2% B-27 supplement minus antioxidants (Invitrogen, USA), 0.25 μg/mL insulin, 10 ng/mL nerve growth factor (NGF) (Invitrogen, USA), 1 mmol/L L-glutamine (Sigma, USA), 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were filtered using a 130-μm filter and counted using a counting chamber. The dissociated DRG cells were then plated at 1×10^5 cells/well in a volume of 0.1 mL for 96-well plates or at 2.5×10^5 -cells/well in a volume of 0.5 mL for 24-well plates (with or without sterile coverslips precoated with poly-L-lysine) (Costar, Corning, NY, USA; Sigma, USA). The clusters were incubated in a humidified atmosphere at 37 °C in a 5% CO_2 incubator for 24 h. To inhibit the growth of non-neuronal cells, the cells were maintained in culture medium containing cytarabine (5 μg/mL) for another 24 h. The cells were incubated in the different experimental conditions for an additional 24 h before observation. For the organotypic DRG culture, each DRG explant was plated at the bottom of a well in a 24-well plate in culture medium directly after removal from the rat embryos. EtOH (1500 mg/dl), Res (0.1, 1, 10, 30 μmol/L), Compound C (CC) (10 μmol/L) or nicotinamide (NCA) (10 mmol/L) was applied as a single bolus directly to the culture media at the same time. The control group was treated with normal culture medium and dimethyl sulfoxide (DMSO, 0.3 μL/mL). To prevent the evaporation of EtOH, the plates were wrapped in Parafilm, and the medium was changed daily.

2.2. Fluorescent labeling and data quantification

The cells on coverslips were rinsed quickly in phosphate buffered saline (PBS) (0.01 mol/L, pH 7.4) to remove the media. Then, the cells were fixed in 4% paraformaldehyde for 40 min followed by 1 h of permeabilization in 3% Triton X-100 at room temperature. After washing in PBS three times, the cells were incubated with 10% normal goat serum for 1 h at room temperature to block nonspecific sites. The mouse monoclonal anti-microtubule-associated protein 2 (MAP2) antibody (1:400, Abcam) was incubated with the samples overnight at 4 °C in a humidified chamber. After washing three times with PBS, the samples were incubated with a goat anti-mouse antibody conjugated to FITC (1:200) for 1 h at room temperature in the dark and then incubated with 4′,6-diamidino-2-phenylindole (DAPI) for an additional 2 min. After that, the cells were immediately coverslipped and anti-fade media applied (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and stored at 4 °C until observation with a fluorescent microscope.

2.3. WST-1 assay for cell viability

After incubation in the different experimental conditions for 24 h, the viability of the DRG neurons in the 96-well plates was detected using a WST-1 (water soluble tetrazolium salt-1, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, China). Briefly, 10 μL of the WST-1-reagent was added to each well, and the intensity of the color formation was detected 4 h later using a Micoplate Photometer (Multiskan MK3, Finland) at a wavelength of 450 nm. The viability of the neurons is expressed as a percentage of the control.

2.4. Determination of neurite outgrowth from DRG explants

The number of nerve fiber bundles extending from the DRG explants was counted after 3 d of culture. Each nerve fiber bundle that extended at least 200 μm from the edge of each DRG explant was counted. Nerve fiber bundles with a length less than 200 μm were ignored in this experiment. Each explant was divided into quarters, and the nerve fiber bundles extending from one quarter of each DRG explant were counted.

2.5. Determination of neuron migration from DRG explants

The migrating neurons were identified as MAP2-IR neurons. MAP2-IR neurons were observed under a fluorescence microscope with a 20 × objective lens. Each explant was divided into quarters, and the MAP2-IR neurons that migrated from one quarter of the edge of each DRG explant were counted as the migrating neurons in each sample.

2.6. Observation of apoptotic neuronal cell death

The nuclear morphological changes of the apoptotic neurons were evaluated by nucleus staining with Hoechst 33342. The DRG neurons grown in a 24-well plate were washed with PBS at 4 °C and fixed with 4% formaldehyde in PBS for 10 min. The neurons were then stained with Hoechst 33342 (10 μg/mL) for 15 min at 37 °C in the dark. After washing three times with PBS, Hoechst 33342 fluorescence was excited with UV wavelengths and assessed under a fluorescent microscope (Olympus, Japan). Under the fluorescent microscope, Hoechst 33342 stained the condensed chromatin in apoptotic cells much more brightly than in normal cells, and cells that showed a shrunken nucleus or condensed or fragmented chromatin were also defined as apoptotic. The ratio of the apoptotic cells to total cells was calculated.

2.7. TUNEL analysis

To detect DNA strand breaks, the cells that had undergone apoptosis were identified with the TMR red *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany), based on the terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate nick-end-labeling (TUNEL) technology. The air-dried cells were fixed for 1 h with 4% paraformaldehyde in PBS at 20 °C, washed with PBS, permeabilized for 2 min on ice in 0.1% Triton X-100 dissolved in 0.1% sodium citrate, and then processed according to the manufacturer's recommendations. Briefly, the TUNEL reaction mixture was added to the samples, and the samples were incubated in a humidified

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