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Research Report

Mitochondrially targeted vitamin E and vitamin E mitigate ethanol-mediated effects on cerebellar granule cell antioxidant defense systems

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Abstract

Ethanol (EtOH) disrupts the structure and function of the developing nervous system, sometimes leading to birth defects associated with fetal alcohol syndrome (FAS). Animal FAS models indicate that cellular membrane peroxidation, intracellular oxidant accumulation, and suppression of endogenous antioxidant enzymes contribute to the toxic effects of EtOH. Mitochondrially targeted vitamin E (MitoVit E), a chemically engineered form of vitamin E (VE) designed to accumulate in the mitochondria, has been shown to inhibit intracellular oxidant accumulation and cell death more effectively than VE. In previous investigations, we have shown that, in vivo, VE reduces neuronal death in the developing cerebellum of EtOH-exposed animals, and, in vitro, VE prevents apoptotic and necrotic death of EtOH-exposed cerebellar granule cells (CGCs). The present investigation shows that, in a FAS CGC model, 1 nM MitoVit E renders significant neuroprotection against EtOH concentrations as high as 1600 mg/dL. The present study also demonstrates that, in this same model, MitoVit E mitigates EtOH-induced accumulation of intracellular oxidants and counteracts suppression of glutathione peroxidase/glutathione reductase (GSH-Px/GSSG-R) functions, protein expression of gamma-glutamylcysteine synthetase (γ-GCS), and total cellular glutathione (GSH) levels. In the presence and absence of EtOH, VE amplifies the protein expression levels of γ-GCS, an enzyme that performs the rate-limiting step for GSH synthesis, and total GSH levels. These results suggest that MitoVit E and VE ameliorate EtOH toxicity through non-oxidant mechanisms–modulations of endogenous cellular proteins–and antioxidant means.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: Mitochondrially targeted vitamin E; Vitamin E; Ethanol; Glutathione; Superoxide dismutase; Gamma-glutamylcysteine synthetase

1. Introduction

As a result of in utero ethanol (EtOH) exposure, fetal alcohol syndrome (FAS) and its partial manifestations affect between 8 and 10 of 1000 newborns per year [15,31]. Over 95% of individuals with full or partial FAS phenotypes have neurological aberrations often related to employment difficulties, cognitive and psychiatric dysfunction, criminal behavior, and the need for assisted-living [37]. Despite aggressive campaigns designed to reduce maternal alcohol abuse during pregnancy, the incidence of FAS has increased approximately seven-fold in the past 15 years. No therapy is available for birth defects caused by fetal alcohol exposure.

Prenatal alcohol exposure adversely affects anatomical and behavioral characteristics through multiple mechanisms. In the nervous system, EtOH has been shown to interfere with various developmental processes (i.e. proliferation, migration, differentiation); increase the production of toxic compounds (e.g. acetylaldehyde, malondialdehyde); pro-

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mote the formation of oxidants (e.g. superoxide [$O_2^{\bullet-}$] hydroxyl [$HO^{\bullet}$] and peroxyl [$LOO^{\bullet}$] radicals, hydrogen peroxide [H_2O_2]); and modulate proteins that affect cell survival (e.g. neurotrophic factors and their receptors, bcl-2 family proteins, caspase family proteases) [7]. EtOH has also been shown to reduce the central nervous system (CNS) concentration of intracellular antioxidants that defend against oxidant attack such as vitamin E (VE), glutathione (GSH), and ascorbate [24,30]. These factors are believed to contribute to EtOH-induced cell damage and death throughout nervous system development.

During the brain growth spurt period, neurons of the cerebellum are most susceptible to EtOH-induced death [8,41]. The brain growth spurt period occurs between postnatal days 4 (P4) and P10 in rodent neonates and during the third trimester of gestation in human fetuses [6]. The primary means of neuronal death during this developmental period appears to be mitochondrially activated apoptosis [43]. Mitochondrial activation of apoptosis due to cellular oxidant accumulation has been well documented [13,24].

Several factors contribute to EtOH-mediated oxidant accumulation [11,19,23,34]. For instance, EtOH initiates the oxidation of mitochondrial and cytoplasmic membrane lipids, damaging membranes and leading to the formation of highly reactive compounds [16,19,33]. Additionally, EtOH dramatically suppresses glutathione peroxidase/glutathione reductase (GSH-Px/GSSG-R) antioxidant defense enzymes [34]. This reduces the capacity of the cell to rid itself of excess lipid peroxides and H_2O_2. In healthy cells, these enzymes regulate lipid peroxides and H_2O_2 levels in the cytoplasm and mitochondria by recycling glutathione (GSH; [25,26]).

In previous studies, we have shown that Pycnogenol®, an antioxidant extract from pine bark, regulates activities of several antioxidant enzymes in a non-oxidant manner. These functional mechanisms likely contribute to the protective capacity of Pycnogenol® against EtOH toxicity [34]. The present investigation was designed to delineate non-oxidant mechanisms of two other antioxidants, mitochondrially activated vitamin E (MitoVit E), a VE derivative chemically engineered to accumulate in the mitochondria, and natural VE. The present study explores the protective capacities and mechanisms of MitoVit E against EtOH insult since MitoVit E abates cell death in other model systems at much lower concentrations that VE [5,12]. Our laboratory has previously shown that VE prevents cell death in FAS cerebellar granule cell (CGC) and animal cerebellar models [9,33].

Using a FAS CGC model, the current investigation uniquely shows that MitoVit E protects against EtOH neurotoxicity and mitigates EtOH suppression of antioxidant enzymes. Specifically, we demonstrate that MitoVit E dose-dependently promotes neuron survival, reduces EtOH-induced oxidant accumulation, affects enzyme activities of the GSH-Px/GSSG-R system, and protects total intracellular GSH levels. The present study also suggests that VE protects against EtOH neurotoxicity in part by enhancing γ-GCS protein expression and total intracellular GSH levels.

2. Materials and methods

2.1. Primary cerebellar granule cell (CGC) culture

Following protocols approved by the Institutional Animal Care and Use Committee (IACUC), CGC cultures were obtained from P9 Long–Evans rats (Charles River Laboratories, Inc., Wilmington, MA). P7–10 CGC cultures provide an excellent model system for exploring mechanisms of EtOH-induced neurotoxicity and antioxidant-mediated neuroprotection since these cultures are: (1) post-mitotic; (2) highly homogenous; (3) EtOH-sensitive; and (4) extensively used to study mechanisms of oxidative stress and apoptosis during development of the CNS, including those induced by EtOH [1,32,33,40,41].

On P9, rat pups were sacrificed and decapitated. Cerebellae were carefully dissected, minced, and suspended in 2 ml Hank's Buffered Salt Solution (HBSS) with 2.5 mg/ml of trypsin (Invitrogen, Carlsbad, CA) and 200 Kunitz units of deoxyribonuclease I (DNase I; Sigma-Aldrich, St. Louis, MO). The trypsin solution was removed and replaced by supplemented Dulbecco's Modified Eagle's Medium (DMEM) pre-warmed to 37 °C. Supplemented DMEM (with low glucose, L-glutamine, pyroxidine HCl, and 110 mg/ml Na+ pyruvate) contained 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin (1×10^4 U/ml)/streptomycin (1×10^4 μg/ml), 0.7% fungizone (amphotericin 250 μg/ml), and 25 mM KCl (all compounds from Sigma). Cells were dissociated by trituration. The pooled suspensions were centrifuged for 10 min (min) at 3000 rpm. The resulting cell pellet was resuspended in 3 ml of fresh medium and filtered using a 20-μm Nitex mesh (Sefar America, Inc, Kansas City, MO). Cells were plated at a density of 1.75×10^5 cells/cm^2 in poly-D-lysine (0.05 mg/ml; Sigma)-coated wells and plates. Experimental cultures were pre-treated with MitoVit E (a generous gift from M.P. Murphy) or natural VE (Sigma). For cell viability assays, cells were pretreated with 1, 10, 25, and 50 nM concentrations of MitoVit E to assess dose-dependent effects of MitoVit E. For oxidant accumulation assays, antioxidant enzyme activity assays, and Western blots, pretreatment concentrations were MitoVit E (50 nM) and VE (50 μM). Cells were established in a 37 °C, 3% CO_2/97% atmospheric air, 100% humidity incubator for 24 hours (h). Cell cultures were determined to be ~95% neuronal by immunochemical detection of glial fibrillary acidic protein (GFAP) and type III β-tubulin.

2.2. EtOH treatment

After 24 h establishment, serum-containing medium was replaced with serum-free modified N2 medium. Modified N2 medium consisted of DMEM/F12 medium at a 3:1 ratio, 5 mM KCl, and 1% N2 supplement. Addition of penicillin/streptomycin and fungizone was equivalent to that of the serum-containing medium. The use of serum-free CGC models in defined N2 media to study EtOH effects on