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Protective effects of resveratrol on the inhibition of hippocampal neurogenesis induced by ethanol during early postnatal life

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ABSTRACT

Ethanol (EtOH) exposure during early postnatal life triggers obvious neurotoxic effects on the developing hippocampus and results in long-term effects on hippocampal neurogenesis. Resveratrol (RSV) has been demonstrated to exert potential neuroprotective effects by promoting hippocampal neurogenesis. However, the effects of RSV on the EtOH-mediated impairment of hippocampal neurogenesis remain undetermined. Thus, mice were pretreated with RSV and were later exposed to EtOH to evaluate its protective effects on EtOH-mediated toxicity during hippocampal development. The results indicated that a brief exposure of EtOH on postnatal day 7 resulted in a significant impairment in hippocampal neurogenesis and a depletion of hippocampal neural precursor cells (NPCs). This effect was attenuated by pretreatment with RSV. Furthermore, EtOH exposure resulted in a reduction in spine density on the granular neurons of the dentate gyrus (DG), and the spines exhibited a less mature morphological phenotype characterized by a higher proportion of stubby spines and a lower proportion of mushroom spines. However, RSV treatment effectively reversed these responses. We further confirmed that RSV treatment reversed the EtOH-induced down-regulation of hippocampal pERK and Hes1 protein levels, which may be related to the proliferation and maintenance of NPCs. Furthermore, EtOH exposure in the C17.2 NPCs also diminished cell proliferation and activated apoptosis, which could be reversed by pretreatment of RSV. Overall, our results suggest that RSV pretreatment protects against EtOH-induced defects in neurogenesis in postnatal mice and may thus play a critical role in preventing EtOH-mediated toxicity in the developing hippocampus.

1. Introduction

Heavy exposure of ethanol (EtOH) in utero may cause neurodevelopmental malformations and results in behavioral and mental deficits, known as fetal alcohol syndrome (FAS) [1–3]. The hippocampus is involved in cognitive processes, such as learning and memory, and is also extremely vulnerable to the neurotoxic effects of EtOH exposure [4–6]. EtOH exposure during the brain growth spurt in both humans (the third trimester) and rodents (first 2 weeks after birth) triggers obvious neurotoxic effects in the developing hippocampus, results in long-term effects on hippocampal neurogenesis, and produces behavioral and cognitive deficits in adults [7,8]. For example, binge-like alcohol exposure during the neonatal period (PD4–9) and a single episode of alcohol exposure on postnatal day 7 (PD7) decreases neurogenesis in the adult hippocampus [7,8]. However, the underlying mechanisms of early alcohol exposure that cause dysfunction in hippocampal neurogenesis remain unclear.

The mammalian subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) is endowed with a pool of neural precursor cells (NPCs) that divide and produce granule cells throughout life [9]. A great majority of the granule cells in the rat DG are generated postnatally at the peak of proliferation occurring near the end of the first postnatal week [10]. Therefore, the size of the NPC pool may be an important determinant of lifetime neurogenesis and hippocampal function. EtOH exposure in postnatal mice at P7 reduced the pool of neural stem cells and progenitor cells in the DG [7]. Meanwhile, chronic alcohol exposure in adolescent nonhuman primates has been reported to significantly decrease the proliferating progenitor pool in the hippocampal DG, thus leading to a lasting effect on hippocampus-associated cognitive tasks [11]. Meanwhile, the plasticity of the neonatal hippocampus allows for the prevention of the teratogenic effects of EtOH

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exposure at an early stage and may be helpful to prevent the occurrence of lifelong brain damage and cognitive dysfunction [12].

Resveratrol (RSV) is a natural stilbene found in the skin of red grapes and in certain medicinal plants [13,14]. RSV has been shown to display multiple neuroprotective effects [15,16]. Treatment with a single dose of RSV after trauma significantly ameliorates trauma-induced hippocampal neuronal loss in rats [17]. In addition, RSV decreases anxiety and increases cortex- and hippocampus-dependent memories in animals subjected to blunt head trauma [18]. RSV has also been recently reported to promote the survival of adult hippocampal NPCs in an animal model of neuroinflammation [19], whereas another study reported that the hippocampal NPCs of intact mice seem to be adversely affected by RSV [20]. Meanwhile, RSV has been demonstrated to decrease brain injury, including injury in the hippocampus, caused by neonatal hypoxic–ischemic [21], and RSV treatment enhanced the generation of newly born neurons in the DG in prenatally stressed rat brains [22]. However, whether RSV can prevent the EtOH-induced exhaustion of the NPC pool in neonatal mice has not yet been examined.

In the present study, mice were pretreated with RSV and were then exposed to EtOH to evaluate the protective effects of RSV against EtOH-mediated toxicity to hippocampal development. We used 5-bromo-2-deoxyuridine (BrdU) and doublecortin (DCX) to investigate the protection of RSV against EtOH-mediated toxicity of hippocampal neurogenesis. In addition, RSV protection against EtOH induced depletion of the NPC pool in the DG was evaluated by double-immunofluorescently stained with SOX2/GFAP, BLBP/Nestin and GFAP/BLBP. The extracellular signal-regulated protein kinase (ERK1/2) belongs to the MAPK family, and has been shown to be involved in the proliferation of NPCs [23]. Hes1 is a downstream target gene in the Notch signaling pathway, which is related to cell proliferation and self-renewal [24]. To determine if ERK1/2 signaling pathways and Hes1 were involved in the proliferation and maintaining of stem/progenitor cells, phosphorylation of ERK1/2 and Hes1 was evaluated by western blot analysis following RSV treatment. Meanwhile, activation of Sirt1 by RSV was also detected with western blot analysis. The multipotent C17.2 NPC line served as an in vitro model to explore the manners of EtOH or RSV on NPC proliferation.

2. Materials and methods

2.1. Animals

Male and female C57/BL6 mice were provided by the Third Military Medical University and were housed in a temperature-controlled room with a standard 12-h light/12-h dark cycle and ad libitum access to food and water. All experimental procedures were approved by Third Military Medical University and were performed according to the guidelines of laboratory animal care and use.

2.2. Drug treatment

The day of birth was designated as postnatal day 0 (P0). On P6, pups were randomly divided into the following four groups: (1) pretreatment with 100% dimethyl sulfoxide (DMSO) followed by normal saline (0.9% NaCl) (DMSO + NS), (2) pretreatment with RSV followed by normal saline (0.9% NaCl) (RSV + NS), (3) pretreatment with DMSO followed by alcohol (DMSO + EtOH), or (4) pretreatment with RSV followed by alcohol (RSV + EtOH). RSV (dissolved in 100% DMSO) was intraperitoneally (i.p.) injected (20 mg/kg) at P6 as previously described [21]. The control groups received an equivalent dose of vehicle DMSO. On P7, animals pretreated with either DMSO or RSV were intraperitoneally (i.p.) injected with a total of 5 g/kg (2.5 g/kg at time of 0 h and 2 h, respectively) EtOH (20% EtOH in sterile normal saline) according to our previously described methods [25]. The controls were injected with an equivalent volume of sterile saline (i.p.). To avoid the litter-effect, the animals in each treatment group were randomly chosen from multiple litters, one pup per litter was selected for each of the group. In this study, we used a minimum number of mice that were required to draw the conclusions and tried to minimize their suffering as much as possible.

BrdU (Sigma-Aldrich) was dissolved in 0.9% saline. To assess cell proliferation in the DG, the pups received one injection of BrdU at a dose of 50 mg/kg per injection (i.p.) on P8 (24 h after the first EtOH injection) and were sacrificed 2 h after the BrdU administration. To analyze neurogenesis in the DG, the pups were administered BrdU (50 mg/kg i.p. four times over 24 h) on P8 and were then sacrificed on P14.

2.3. Immunohistochemistry and immunofluorescence

The brain was dissected and fixed in 4% paraformaldehyde (PFA) for 24 h at 4 °C. For paraffin sections, tissues were processed for paraffin embedding, and coronal sections (5 μm thick) were collected. The remaining brains were post-fixed in a 30% sucrose solution with 4% PFA at 4 °C, and coronal cryosections (15 μm thick at P8, 20 μm thick at P14) were collected. Paraffin sections were used for hematoxylin–eosin (HE) staining to examine the cytoarchitecture of the hippocampus. Immunohistochemistry was performed according to Yang et al. [25]. In brief, sections were exposed to the following primary antibodies in 1% BSA (12 h, 4 °C): anti-Nestin (1:400) (BD Biosciences), anti-BLBP (1:400), anti-Sox2 (1:500) and anti-glial fibrillary acidic protein (GFAP) (1: 100) (Chemicon, Temecula, CA, USA), and 1% BSA replaced primary antibodies in negative controls. For BrdU staining, all the sections were pretreated with 2 N HCl for 1 h at 37 °C to denature the DNA followed by 0.1 M borax (pH 8.5) treatment for 10 min to neutralize before the regular immunostaining procedure. After washing the samples in PBS for BrdU staining, the sections were incubated with biotin-conjugated secondary antibody and were visualized under bright-field microscopy with a diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA, USA). For immunofluorescence, the sections were then incubated with Cy3- or 488-conjugated (both at 1:400, 3 h; Jackson ImmunoResearch, West Grove, PA, USA) secondary antibodies and mounted in Vectashield (Vector). Nuclei were subsequently stained with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime, China). The stained cells were viewed and photographed under a Zeiss (Oberkochen, Germany) Axivert microscope equipped with a Zeiss AxioCam digital color camera connected to the Zeiss AxioVision 3.0 system.

2.4. Western blotting

Hippocampi were isolated and homogenized in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). After centrifugation of lysates (15,000 *g*, 5 min at 4 °C), the protein concentration was determined via the Bicinchoninic Acid Kit (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples (40 μg per lane) were separated on a 12% SDS-polyacrylamide gel and then transferred onto polyvinylidene fluoride (PVDF) membranes for immunoblotting. The membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and 5% fat-free milk for 1 h at RT. The membranes were then incubated (overnight at 4 °C) with rabbit antibodies against Hes1 (1:1000, Chemicon), p-ERK1/2 or total-ERK1/2 (1:1000, Cell Signaling), Sirt1 (1:1000, Chemicon) and β-actin (1:2000, Cell CWBIO, Beijing, China), followed by 1 h of RT incubation with a peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:2000; Santa Cruz Biotechnology). All western blotting data were representative of at least three independent experiments. Specific protein bands on the membranes were visualized by the enhanced chemiluminescence method (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions. The relative intensities of Hes1, Sirt1 and p-ERK were normalized to the internal