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ACTIVITY-DEPENDENT NEUROTROPHIC FACTOR-DERIVED PEPTIDE PREVENTS ALCOHOL-INDUCED APOPTOSIS, IN PART, THROUGH BCL2 AND C-JUN N-TERMINAL KINASE SIGNALING PATHWAYS IN FETAL BRAIN OF C57BL/6 MOUSE

Y. SARI,[a]* J. M. WEEDMAN[b1] AND S. GE[a]

[a]*Department of Pharmacology, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, 3000 Arlington Ave. Toledo, OH 43614, USA*

[b]*Indiana University Bloomington, 10th Street. Bloomington, IN 47405*

Abstract—Fetal alcohol exposure is known to induce alteration in fetal brain development. In this study, we focused on neuroprotection against the effects of alcohol exposure using ADNF-9, a peptide derived from activity-dependent neurotrophic factor. We used a mouse model of fetal alcohol exposure to identify the intracellular mechanisms underlying the neuroprotective effects of ADNF-9. On embryonic day 7 (E7), weight-matched pregnant females were assigned to the following groups: (1) ethanol liquid diet (ALC) of 25% (4.49%, v/v) ethanol-derived calories; (2) pair-fed control (PF); (3) ALC combined with administration (i.p.) of ADNF-9 (ALC/ADNF-9); and (4) pair-fed combined with administration (i.p.) of ADNF-9 (PF/ADNF-9). On E13, fetal brains were collected, weighed, and apoptosis was determined using TdT-mediated dUTP nick-end labeling (TUNEL) assay. Bcl2 protein and phospho-c-Jun N-terminal kinase (JNK) levels were determined using Western blot and enzyme immunometric assay, respectively. ADNF-9 administration significantly prevented alcohol-induced reductions in fetal brain weight. In addition, ADNF-9 prevented an alcohol-induced increase in cell death in the primordium of the cerebral cortex and ganglionic eminence. Western blot analysis of the mitochondrial protein fractions revealed that ADNF-9 administration prevented an alcohol-induced reduction in the Bcl2 level. Moreover, an analysis of the proteins in the upstream signaling pathway revealed that ADNF-9 downregulated the phosphorylation of JNK. These data indicate that the mitochondrial Bcl2 pathway and JNK upstream signaling pathway are the intracellular targets of ADNF-9. The neuroprotective mechanism of action of ADNF-9 provides a direction for potential therapeutics against alcohol-induced neural damage involving mitochondrial dysfunction. Published by Elsevier Ltd on behalf of IBRO.

Key words: ADNF-9, neuroprotection, JNK, mitochondria, apoptosis, Bcl2.

[1] Present address: Indiana University School of Medicine, Indianapolis, IN, USA.

*Corresponding author. Tel: +1-419-383-1507; fax: +1-419-383-1909.
E-mail address: youssef.sari@utoledo.edu (Y. Sari).

Abbreviations: ADNF, activity-dependent neurotrophic factor; ALC, alcohol; BAC, blood alcohol concentration; BAD protein, Bcl2-associated agonist of cell death protein; EDC, ethanol-derived calories; EDTA, ethylenediaminetetraacetic acid; EIA, enzyme immunometric assay; FAE, fetal alcohol exposure; FAS, fetal alcohol syndrome; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; JNK, c-Jun N-terminal kinase; PBS, phosphate-buffered saline; PF, Pair-fed control group; TUNEL, TdT-mediated dUTP nick-end labeling.

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Alcohol consumption during pregnancy is known to alter the development of the central nervous system. Brain growth deficits and behavioral abnormalities are two of the pathological features of fetal alcohol syndrome (FAS) and fetal alcohol exposure (FAE) (Mattson et al., 1992, 1994, 1996a,b; Roebuck et al., 1998). The effects of prenatal alcohol exposure in brain growth are associated with apoptosis (Ikonomidou et al., 2000). Using our established liquid diet paradigm, which mimics human drinking behavior, we demonstrated that prenatal alcohol exposure from embryonic day 7 (E7) to E13 induced an increase in apoptosis or cell death (Sari, 2009; Sari et al., 2009). The increases in cell death were associated with increases in caspase-3 activation and cytosolic cytochrome c. Importantly, we revealed that prenatal alcohol exposure reduced the level of mitochondrial cytochrome c (Sari, 2009; Sari et al., 2009). Recent studies from our laboratory demonstrated that prenatal alcohol exposure induced the down-regulation of several key mitochondrial proteins including enzymes and carrier proteins (Sari et al., 2010b).

Although less is known about the treatment or prevention, studies performed by us and others have demonstrated the possible prevention of the effects of prenatal alcohol exposure using derived peptides in animal models (Spong et al., 2001; Chen et al., 2005; Sari and Gozes, 2006; Parnell et al., 2007; Zhou et al., 2008; Sari, 2009; Sari et al., 2009) and *in vitro* (Wilkemeyer et al., 2004; Chen et al., 2005; Zhang et al., 2005; Pascual and Guerri, 2007; Chen and Charness, 2008). Among these peptides are ADNF-9 peptide with sequence SALLRSIPA, also known as SAL, which is derived from an activity-dependent neurotrophic factor (ADNF) (Brenneman and Gozes, 1996; Brenneman et al., 1998) and peptide with sequence, NAPVSIPQ, also known as NAP, which is derived from an activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999; Zamostiano et al., 2001). In addition, we have recently identified a new synthesized peptide, colivelin, that appears to play a key neuroprotective role in the FAE model (Sari et al., 2009). Colivelin is composed of ADNF-9 and AGA-(C8R)HNG17 (PAGASRLLLLTGEIDLP).

In this study, we focused on testing ADNF-9 as a neurotrophic peptide against the insult of prenatal alcohol exposure in a fetal mouse brain. We tested the D-form of this peptide since it has been shown to exhibit high protective potency to the fetuses and is suggested to be more stable than the L-form (Brenneman et al., 2004). Experiments conducted in our laboratory revealed the effects of

the protective properties of the D-ADNF-9 peptide against fetal alcohol-related brain growth restriction and fetal alcohol-induced increases of apoptosis in the FAE model [for review see ref. (Sari and Gozes, 2006)]. Thus, in this study, we have tested the D-ADNF-9 form as a potent neuroprotective peptide in the fetal brain exposed prenatally to alcohol from E7 to E13. We have investigated if ADNF-9 protects against alcohol-induced reduction in fetal brains and increases in apoptosis at E13. Importantly, we have determined the neuroprotective effects of ADNF-9 involving mitochondrial intrinsic and extrinsic pathways.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 mice were used in this study. This mouse model is an established and well studied model in the field of FAE and FAS (Webster et al., 1983; Spong et al., 2001; Sari and Gozes, 2006; Sari, 2009; Sari et al., 2009, 2010a,b). The mice can consume freely ethanol liquid diet or pair-fed liquid diet. The mice were supplied by Harlan, Inc. (Indianapolis, IN, USA). Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Toledo and Indiana University and are in accordance with the guidelines of the Institutional Animal Care and Use Committee at the National Institutes of Health and The Guide for the Care and Use of Laboratory Animals.

Breeding and treatments

The female mice were placed in the male home cage for 2 h. The females were then checked for a sperm plug by a vaginal smear. E0 was designated as the time point when the vaginal smear was positive. Weight-matched pregnant females at E7 were assigned to the following groups: (1) ethanol liquid diet group (ALC, $n=10$ litters), which was fed with chocolate sustacal (supplemented with vitamins and minerals) liquid diet of 25% (4.49%, v/v) ethanol-derived calories (EDC), and simultaneously i.p. injected saline vehicle; (2) pair-fed control group (PF, pair-fed to ethanol-fed group, $n=12$ litters), which was fed with a maltose-dextrin solution isocaloric to the dose of ethanol used, and simultaneously i.p. injected saline vehicle; (3) pair-fed combined with administration (i.p.) of ADNF-9 (PF/ADNF-9, 30 μg/20 g of body weight, $n=6$ litters); and (4) treatment group, which received an ADNF-9 i.p. injection alongside alcohol exposure in liquid diet (ALC/ADNF-9, 30 μg/20 g, $n=8$ litters). The PF group dam, yoked individually to an ALC or ALC/ADNF-9 dam, was given daily amounts of a matched isocaloric liquid diet with maltose-dextrin substituted for the ethanol at all times during gestation (E7–E13). The pregnant mice had continuous, 24-h access to the alcohol diet or PF liquid diet for 7 days.

The fortified liquid diet contained 237 ml of chocolate-flavored sustacal (CVS Pharmacy), 1.44 g of vitamin diet fortification mixture, and 1.2 g of Salt Mixture XIV. For the ethanol diet, 15.3 ml (4.49% v/v; 25% EDC) of 95% ethanol was added to the fortified sustacal formula with water that was added to make 320 ml of diet with 1 cal/ml (ethanol). For the isocaloric control diet, 20.2 g of maltose dextrin was added to the fortified sustacal formula with water that was added to bring it to 1 cal/ml. One day before treatment, the ALC and PF dams were adapted to the liquid diet 9:00 and 10:00 h, the dams were weighed, the volume of liquid diet consumed during the previous 24 h was recorded from 30-ml graduated screw-cap tubes, and freshly prepared diet was provided. The PF and PF/ADNF-9 litters had limited access to the EDC liquid diet each day to match the drinking of ALC and ALC/ADNF-9 litters, respectively. The average of consumed alcohol or pair-fed liquid diet by all the litters (ml of consumed diet/g of body weight) for the 7-day period for each group is as follows: PF (0.688 ± 0.011, $n=12$); PF/ADNF-9 (0.698 ± 0.011, $n=6$); ALC (0.680 ± 0.009, $n=10$); and ALC/ADNF-9 (0.695 ± 0.012, $n=8$).

The blood alcohol concentration (BAC) was performed in a separate group of pregnant C57BL/6 mice exposed prenatally to 25% (4.49%, v/v) EDC as described previously (Zhou et al., 2003). In this later study, using similar drinking paradigm, extracted plasma samples were analyzed using Analox Alcohol Analyzer for the determination of the ethanol concentrations in blood. The BACs were as follows: $=44.3\pm11.6$ mg/dl on E8 and $=54.7\pm14.2$ mg/dl on E11 (Zhou et al., 2003). Although our present study was focused at E13 stage, the BAC measured at E14 was $=142.7\pm49.5$ mg/dl (Zhou et al., 2003).

Fetal brains

Pregnant mice were deeply anesthetized with a CO_2 procedure followed by a cervical dislocation at E13, and the fetuses were removed. The experimenter involved in the fetal brain dissections was blind to the treatment groups. The fetal brains were further dissected from the base of the primordium olfactory bulb to the base of the metencephalon. From the same litter, five fetal brains from each litter were randomly selected, weighed, and quickly frozen and stored at -70 °C until used for chemical assays; and the other fetal brains from each litter were fixed in 4% paraformaldehyde for the detection of TdT-mediated dUTP nick-end labeling (TUNEL)-positive cells. The average fetal brain weights from each litter is reported as one "n" value and was used for statistical analysis. The number of animals per group used for statistical analysis of fetal brain weight is as follows: PF ($n=12$ litters), PF/ADNF-9/30 ($n=6$ litters), ALC ($n=10$ litters), and ALC/ADNF-9/30 ($n=8$ litters).

Mitochondrial protein extraction

One frozen fetal brain from each litter of control or treatment group was selected randomly for mitochondrial protein extraction. Frozen fetal brains were homogenized for 30 min with digitonin (0.05%) in a lysis buffer (250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and 1:100 protease cocktail inhibitor). The homogenates were then centrifuged for 12 min (13,000 rpm, 4 °C). The supernatant (cytosolic fraction) was removed and stored at -80 °C. The pellet was resuspended in a second lysis buffer for 30 min (133 mM NaCl, 50 mM Tris pH 8.0, SDS 0.1% [w/v], sodium deoxycholate 0.5% [w/v], Igepal CA630 1.0% [v/v], and protease cocktail inhibitor 1:100). The mixture was then centrifuged, and the supernatant (mitochondrial fraction) was collected and stored at -70 °C.

Western blot for detection and measurement of the levels of Bcl2 proteins

Western blots were performed in mitochondrial fraction from the PF, PF/ADNF-9, ALC, and ALC/ADNF-9 groups. Mitochondrial proteins were separated in a 10–20% glycine gel (Invitrogen). Proteins were then transferred onto a nitrocellulose membrane electrophoretically at 30 V for 1 h. The membranes were then blocked using 3% milk in TBST (50 mM Tris–HCl; 150 mM NaCl, pH 7.4; 0.1% Tween 20) for 30 min at room temperature. The membranes were then incubated with a mouse anti-Bcl2 antibody (BD Transduction laboratories) at a 1:1,000 dilution in a blocking buffer at 4 °C. After washing and blocking, the membranes were incubated with a horseradish peroxidase (HRP)-labeled anti-mouse IgG secondary antibody (1:5,000 dilution) in a blocking buffer. Protein loading was normalized using β-tubulin antibody immunoblotting as a loading control. Chemiluminescent detection of HRP (SuperSignal West Pico; Pierce) was followed by exposure of the membranes to Kodak BioMax MR film (Thermo Fisher Scientific). The film was developed on a SRX-101A machine. Digitized images of immunoreactive proteins were quantified using an MCID system.