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ACTIVITY-DEPENDENT NEUROPROTECTIVE PROTEIN–DERIVED PEPTIDE, NAP, PREVENTING ALCOHOL-INDUCED APOPTOSIS IN FETAL BRAIN OF C57BL/6 MOUSE

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Abstract—Possible prevention of the effects of prenatal alcohol exposure has been investigated using peptides that were previously shown to be involved in neuroprotection both *in vitro* and *in vivo*. I focused in this study on investigating the neuroprotective effects of one of these peptides with regard to the determination of the downstream signaling pathways involved in neuroprotection. This peptide with the sequence NAPVSIPQ, known as NAP, a fragment of activity-dependent neuroprotective protein, demonstrated a potent protective effect against oxidative stress associated with alcohol exposure. On embryonic day 7 (E7), weight-matched C57BL/6 pregnant females were assigned the following groups: (1) Ethanol liquid diet group (ALC) 25% (4.49%, v/v) ethano-derived calories, (2) Pair-fed (PF) control group (3) Chow control group, (4) treatment groups with alcohol alongside i.p. injections of D-NAP (ALC/D-NAP, 20 or 30 μg/20 g body weight), (5) PF/D-NAP control group. On E13, fetal brains were collected and assayed for TdT-mediated dUTP nick end labeling (TUNEL) staining, caspase-3 colorimetric assay and ELISA for cytochrome c detection. My results show that NAP significantly prevented alcohol-induced weight reduction of the fetal brain. Apoptosis was determined by TUNEL staining; NAP administration significantly prevented alcohol-induced increases in TUNEL-positive cells in primordium cingulate cortex and basal ganglia eminence. The investigation of downstream signaling pathways involving NAP neuroprotection revealed that this peptide significantly prevented alcohol-induced increase in the concentrations of caspase-3 in E13 fetal brains. Moreover, ELISA for cytochrome c shows that NAP significantly prevented both alcohol-induced increases in the level of cytosolic cytochrome c and alcohol-induced decreases in the level of mitochondrial cytochrome c. These data provide an understanding of NAP intracellular target, and the downstream mechanisms of action that will pave a path toward potential therapeutics against alcohol intoxication during prenatal stages.

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Abbreviations: ADNP, activity-dependent neuroprotective protein; ALC, ethanol liquid diet group; ANOVA, analysis of variance; Apaf-1, apoptotic protease activating factor 1; BAC, blood alcohol concentration; E, embryonic day; EDC, ethanol derived calories; EDTA, ethylenediaminetetraacetic acid; FAE, fetal alcohol exposure; FAS, fetal alcohol syndrome; NIH-NICHD, National Institutes of Health–National Institute of Child Health and Human Development; OD, optical density; PF, pair-fed control group; RT, room temperature; TUNEL, TdT-mediated dUTP nick end labeling.

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Brain growth restriction is a hallmark of fetal alcohol exposure (FAE) and fetal alcohol syndrome (FAS) models (Roebuck et al., 1998). Alcohol exposure impedes cerebrum and cerebellum growth (Bauer-Moffett and Altman, 1975, 1977; Kornguth et al., 1979; Samson and Diaz, 1981; Sulik et al., 1981; Barron et al., 1988; Bonthius and West, 1990; Sari and Gozes, 2006). Our and other studies have demonstrated that the effects of prenatal alcohol exposure depend on the dose and timing of alcohol treatment (Webster et al., 1983; Sari and Gozes, 2006). During the period between embryonic day 7 (E7) and E14, the developing brain exhibited the highest susceptibility to alcohol exposure. Deficits in fetal brain growth might be induced possibly through an apoptotic mechanism (Ikonomidou et al., 2000). Mitochondrial activation of apoptotic pathways is one of the major players in ethanol-mediated neuronal death. Although studies have demonstrated that prenatal alcohol exposure induces mitochondrial dysfunction, little is known about its signaling pathways. The mitochondrion is a target organelle in ethanol-induced organ-system toxicity (Hoek et al., 2002). Prenatal alcohol exposure–induced mitochondrial dysfunction includes decreased mitochondrial glutathione concentration, decreased activities of respiratory chain complex IV and ATP synthase, and increased mitochondrial permeability transition (Ramachandran et al., 2001; Spong et al., 2001; Xu et al., 2005a; Green et al., 2006). We have recently found that prenatal alcohol exposure induced decreases in ATP synthase, ADP/ATP translocase, NADH dehydrogenase, ubiquinol-cytochrome-c reductase and prohibitin at E13 (Sari and Mechref, 2008). Together, these findings indicate that mitochondrial dysfunction is a key factor in alcohol-induced apoptosis.

E13 age was chosen as an end-point of alcohol exposure because this developmental age holds several interests in my ongoing research projects in FAE model. Most of the developmental regulatory proteins are highly expressed at this age of development and altered as a consequence of alcohol exposure (Sari and Gozes, 2006; Sari and Mechref, 2008). In addition, at E13, the neural tube has undergone five major divisions to form a fetal brain composed of telencephalon, diencephalon, midbrain, hindbrain and spinal cord. Moreover, at this developmental stage, the 5-HT neurotransmitter system has formed and initiated its differentiation. Our previous study reported that prenatal alcohol exposure induces neural tube defects

early at E13 (Zhou et al., 2003) and similarly alters early 5-HT neurons [for review see ref. (Sari and Gozes, 2006)].

Studies investigating alcohol exposure during development in animal models may provide important information for the identification of possible mechanisms of neuroprotection and allow for the development of intervention procedures, which may protect or attenuate the deleterious effects of alcohol exposure during pregnancy. Several studies have investigated the possible prevention of the effects of prenatal alcohol exposure by the treatment of pregnant mice with peptides that have been shown to be involved in neuroprotection (Spong et al., 2001; Poggi et al., 2003; Brenneman et al., 2004; Zhou et al., 2004; Sari and Gozes, 2006). Among these peptides, SALLRSIPA, known as SAL or ADNF-9, is derived from activity-dependent neurotrophic factor (ADNF) (Brenneman and Gozes, 1996; Brenneman et al., 1998). Another peptide with the sequence NAPVSIPQ peptide, termed NAP, derived from activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999; Zamostiano et al., 2001), demonstrated a potent protective effect against oxidative stress.

In the present study, using our established FAE model in C57BL/6, we tested the neuroprotective effects of NAP at early stage of development E13. The ADNP is released from astroglia (Bassan et al., 1999) and found to be regulated by vasoactive intestinal peptide, VIP (Furman et al., 2004). High ADNP mRNA expression was found in the hippocampus, cerebral cortex and cerebellum in the mouse (Bassan et al., 1999) and in the human brain (Zamostiano et al., 2001), indicating a potential role in the central nervous system (Gozes et al., 1999). It has been further shown that knocking out the ADNP gene is lethal to the embryo and the ADNP-deficient embryos die at the time of neural tube closure (Pinhasov et al., 2003).

Our and other studies have focused on the role of NAP in neuroprotection against prenatal alcohol exposure [for review see ref. (Sari and Gozes, 2006)]. A recent study demonstrated a significant decrease in the ADNP mRNA in the cerebral cortex of 7-day-old pups that were exposed prenatally to alcohol (Pascual and Guerri, 2007). Using an established model for FAS, it has been demonstrated at National Institutes of Health–National Institute of Child Health and Human Development (NIH-NICHD) that pretreatment with NAP prevented the fetal death and abnormalities induced by prenatal alcohol exposure (Spong et al., 2001). Importantly, the effects of enantiomer conformation (L- and D-forms) of this peptide against the insult of alcohol exposure was also investigated in FAS model (Brenneman et al., 2004). The D-form of NAP resulted in a potent effect with no loss of protective response regardless of increases in the doses.

We have demonstrated previously the effects of D-NAP for its protective properties against fetal alcohol–related brain growth restriction in FAE model in C57BL/6 mice (Sari et al., 2001, 2003; Sari and Zhou, 2004; Zhou et al., 2004; Sari and Gozes, 2006). Administration of D-NAP alongside alcohol exposure, from E7 to E15, antagonized the effects of alcohol on brain weight, the size of several forebrain regions, and neural tube development [recently reviewed by us (Sari and Gozes, 2006)]. In the present study, using a similar liquid diet drinking paradigm, we investigated the cellular mechanism of NAP in neuroprotection with more focus on the apoptotic mechanisms that may involve extrinsic and intrinsic mitochondrial signaling pathways at E13.

EXPERIMENTAL PROCEDURES

Synthesis of D-NAP peptide

The peptide D-NAP (NAPVSIPQ, all amino acids are D form) was synthesized in-house at Indiana University using a standard solid-phase Fmoc chemistry on a ABI 433 peptide synthesizer. The peptide was de-protected/cleaved from the resin in TFA cocktail and purified by HPLC. The purity of the final compound was >98% as identified with analytical HPLC. The mass of D-NAP was determined to be m/z 825.3$[M+H]^+$ (calc.825.4) by ESI-MS.

Animals

Mice C57BL/6 were used in these studies. Both male and female mice were obtained at 6–7 weeks of age from Harlan Laboratory at Indianapolis, IN, USA. All mice were housed in the departmental animal colony in a vivarium with a controlled climate (temperature 22 °C, 30% humidity) with a 12-h light/dark cycle. Animals used in these procedures were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Indiana University Bloomington and are in accordance with the guidelines of the Institutional Animal Care and Use Committee of the NIH, and the Guide for the Care and Use of Laboratory Animals. Pregnants mice were euthanized by CO_2 followed by physical translocation. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. In addition the number of animals used in this study was minimized according to our previous studies and power analyses.

Breeding and treatment procedure

Female mice were placed into male home cages for 2 h and were then checked for a sperm plug by vaginal smear immediately afterward. When the plugs were positive, we designated this time point as E0. On E7, weight-matched pregnant females were assigned to the following groups: (1) Ethanol liquid diet group (ALC, n=7) fed with chocolate Sustacal (supplemented with vitamins and minerals, Bio-Serv, Frenchtown, NJ, USA) liquid diet 25% (4.49%, v/v) ethanol derived calories (EDC), (2) Pair-fed control group (PF, pair-fed to ethanol-fed group, n=7) fed with a maltose–dextrin solution which was made isocaloric to the dose of ethanol used, (3) Chow group fed with mouse chow pellets and water (Chow, n=5), (4) treatment group, i.p. injections of D-NAP alongside alcohol exposure (ALC/NAP20, 20 μg/20 g body weight, n=6), (5) treatment group, i.p. injections of D-NAP alongside alcohol exposure (ALC/NAP30, 30 μg/20 g body weight, n=5), and (6) PF/NAP control group (D-NAP i.p. injected at a dose of 30 μg/20 g body weight, n=5). NAP was administered once a day from E7 through E13. The PF, PF/NAP, and Chow served as control groups. We have generated fetal brains from 35 pregnant mice (Chow, PF, PF/NAP, ALC, ALC/NAP20 and ALC/NAP30) at E13.

I used my established liquid diet mixed with alcohol in FAE model that mimics moderate alcohol drinking [for review see (Sari and Gozes, 2006)]. As published by Middaugh and colleagues (Middaugh et al., 1988; Middaugh and Boggan, 1995), the fortified liquid diet contained 237 ml of chocolate-flavored Sustacal (CVS Pharmacy), 1.44 g Vitamin Diet Fortification Mixture and 1.2 g Salt