Prevention of Fetal Demise and Growth Restriction in a Mouse Model of Fetal Alcohol Syndrome

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Received October 17, 2000; accepted January 2, 2001. This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Two peptides [NAPVSIPQ (NAP) and SALLRSIPA (ADNF-9)], that are associated with novel glial proteins regulated by vasoactive intestinal peptide, are shown now to provide protective intervention in a model of fetal alcohol syndrome. Fetal demise and growth restriction were produced after intraperitoneal injection of ethanol to pregnant mice during midgestation (E8). Death and growth abnormalities elicited by alcohol treatment during development are believed to be associated, in part, with severe oxidative damage. NAP and ADNF-9 have been shown to exhibit antioxidative and antiapoptotic actions in vitro. Pretreatment with an equimolar combination of the peptides prevented the alcohol-induced fetal death and growth abnormalities. Pretreatment with NAP alone resulted in a significant decrease in alcohol-associated fetal death; whereas ADNF-9 alone had no detectable effect on fetal survival after alcohol exposure, indicating a pharmacological distinction between the peptides. Biochemical assessment of the fetuses indicated that the combination peptide treatment prevented the alcohol-induced decreases in reduced glutathione. Peptide efficacy was evident with either 30-min pretreatment or with 1-h post-alcohol administration. Bioavailability studies with [3H]NAPVSIPQ indicated that 39% of the total radioactivity comigrated with intact peptide in the fetus 60 min after administration. These studies demonstrate that fetal death and growth restriction associated with prenatal alcohol exposure were prevented by combinatorial peptide treatment and suggest that this therapeutic strategy be explored in other models/diseases associated with oxidative stress.

In the United States, fetal alcohol syndrome (FAS) occurs in 0.5 to 3 births per 1000 births each year (Stratton et al., 1996). During the 1990s, the prevalence of alcohol consumption, including binge drinking, has increased among pregnant women, whereas there has been little change in non-pregnant women of child-bearing age (Ebrahim et al., 1999). Maternal alcohol consumption is the most commonly identifiable nongenetic cause of mental retardation (Windham et al., 1997). In addition, consuming more than three drinks per week in the first trimester has been shown to double the risk of miscarriage (Jimenez et al., 1993). Children with FAS, however, do not preclude assigning the diagnosis. Children with fetal alcohol exposure without FAS can have serious mental and learning impairments and by magnetic resonance imaging have evidence of structural brain defects (Mattson et al., 1996).

The purpose of the present study was to attempt an intervention against alcohol-induced damage by treating pregnant mice with peptides that have been shown to be neuroprotective in dissociated cerebral cortical cell culture (Brenneman et al., 1998; Bassan et al., 1999) and in vivo (Bassan et al., 1999; Gozes et al., 2000). In the adult, there are documented interactions between alcohol and vasoactive intestinal peptide (VIP) (Gressens et al., 1992; Madeira et al., 1997), a neuropeptide that is a regulator of early postimplantation mouse embryonic growth (Spong et al., 1999). In addition, VIP antagonist treatment of pregnant mice results in some of the features of FAS, including fetal growth restriction and microcephaly (Gressens et al., 1994). The rationale for the use of these peptides resides in the neuroprotective (Brenneman and Eiden, 1986; Brenneman et al., 1988) and growth-promoting (Gressens et al., 1993) actions of VIP. These actions of VIP are mediated directly through the

ABBREVIATIONS: FAS, fetal alcohol syndrome; VIP, vasoactive intestinal peptide; ADNF, activity-dependent neurotrophic factor; ADNF-9, SALLRSIPA; ADNP, activity-dependent neuroprotective protein; NAP, NAPVSIPQ; DPBS, Dulbecco’s phosphate-buffered saline; GSH, reduced glutathione; GSSG, oxidized glutathione; ANOVA, analysis of variance.
release of glia-derived substances (Brenneman et al., 1987), including activity-dependent neurotrophic factor (ADNF), a protein that has growth-promoting (Glazner et al., 1999b), antiapoptotic (Gozes et al., 1997), and antioxidative properties (Brenneman and Gozes, 1996). Analysis of digest peptides of purified ADNF revealed active peptide fragments (Brenneman and Gozes, 1996). Structure-activity analysis of an active peptide fragment demonstrated that a nine amino acid peptide (SALLRSIPA) was the shortest sequence that retained the potent, protective properties of ADNF (Brenneman et al., 1998). This peptide was named ADNF-9 because it was comprised of nine amino acids and captured the survival-promoting activity of ADNF.

Antibodies against ADNF-9 were used to clone and identify a functionally related protein: activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999; Zamostiano et al., 2001). An ADNF-9-like fragment of ADNP [NAPVSIPQ (NAP)] was discovered that protected against oxidative stress (Offen et al., 2000; Steingart et al., 2000). ADNF-9 and NAP exhibit remarkable potency and neuroprotective activity in animal models related to neurodegeneration, including apolipoprotein E-deficient mice (Bassan et al., 1999), rats treated with a cholinotoxin (Gozes et al., 1997), and mice subjected to closed-head injury (Beni-Adani et al., 2001). Thus, ADNF-9 and NAP were chosen for evaluation in a FAS model.

The model for FAS employed in the present study is based on an acute high exposure to alcohol that provided an outcome emphasizing the severity of alcohol-induced growth restriction and fetal death (Webster et al., 1980). Since treatment on gestational day 8 resulted in the highest rate of fetal anomalies and demises (Webster et al., 1980), and VIP's growth-regulating effects on the embryo are limited to the early postimplantation period (Gressens et al., 1993; 1994), day 8 was chosen as the optimal and most severe test for the protective activity of the peptides. For accurate and reproducible administration of alcohol and peptide, a model utilizing intraperitoneal injection was chosen (Webster et al., 1980). The highest alcohol dose in the model (0.03 ml/kg) was selected to provide the most severe test of efficacy. Previous studies indicated that the intraperitoneal model results in higher blood alcohol concentrations than that obtained by an oral route (Webster et al., 1980), providing a stringent test to evaluate treatment efficacy.

Materials and Methods

Animals. C57-B16J female mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were kept under a 12-h light/dark regimen, with food and water available at all times. The mice received humane animal care in compliance with the Guideline for Care and Use of Experimental Animals. Six-week-old females (21–24 g) were mated with C57-B16J males for 4 h. The presence of a vaginal plug was considered day 0 of pregnancy.

Treatment Groups. A well delineated model for FAS was used (Webster et al., 1980). Animals were injected (intraperitoneal; i.p.) on gestational day 8 with 25% ethyl alcohol in saline (v/v) or vehicle alone at 0.030 ml/g of body weight at 9 AM. Pretreatment (i.p.) with VIP and ADNP/ADNF peptides (NAP, ADNF-9, NAP + ADNF-9) were given 30 min before alcohol. Dosages of the peptides were NAP (20 and 40 μg), ADNF-9 (20 μg), NAP (20 μg) + ADNF-9 (20 μg), and VIP (1 μg). NAP + ADNF-9 without alcohol was also studied. NAP was diluted in 50 μl of dimethyl sulfoxide and diluted in filtered Dulbecco's phosphate-buffered saline (DPBS). ADNF-9 was dissolved and diluted in filtered DPBS. VIP was dissolved in 20 μl of acetic acid (0.02%) and diluted in water and DPBS. Since the animals receiving alcohol were incapacitated for approximately 6 h following injection, food and water were withheld from all groups for the initial 6 h to allow accurate assessment of fetal weights.

Evaluation of Litter Size and Pup Weights. Fetal brain and body weights were obtained on pregnancy day 18. The number of pups and fetal deaths/resorption were counted. The number of live pups was calculated as a percentage of the total litter size. The litter mean was used as the N for comparisons of fetal survival, pup weight, and brain weight.

Bioavailability. The bioavailability of NAP to embryos was assessed by intraperitoneal administration of [3H]NAP (3 μCi/20 μg of NAP/injection) to pregnant mice at E8. Before labeling with tritium, NAPVSIPQ was synthesized with one modification: replacing the proline in the third position with a dihydroproline (SynPep Corp., Dublin, CA). The NAPVSIPQ [propyl 3,3,4-3H] was custom-labeled by American Radiolabeled Chemicals, Inc. (St. Louis, MO). The specific activity of the [3H]NAP was 50 μCi/mmol, and the labeled peptide was dissolved in ethanol and stored at ~80°C. The purity of the labeled NAP was >99.5% as determined by high-performance liquid chromatography with a Vydac C18 column (4.6 × 250 mm). Tissues were collected 30 and 60 min after injection into 300 μl of 0.1 N HCl. Samples were homogenized with disposable pellet pestles and centrifuged at 10,000g (10 min at 4°C). The supernatant was frozen on dry ice and stored at ~80°C until analyzed by size exclusion analysis (Superdex Peptide HR 10/30; Amersham Pharmacia Biotech, Piscataway, NJ) on a fast performance liquid chromatograph.

Reduced (GSH) and Oxidized (GSSG) Glutathione Levels. GSH and GSSG were simultaneously detected and measured with capillary electrophoresis in embryo/decidua 8 h after treatment using a previously described method (Muscarli et al., 1998). NAP + ADNF-9 was given 30 min before alcohol. Embryo/decidual samples were pooled in groups of two; at least eight samples were run per treatment. Identity of GSH and GSSG was made in relation to the migration of the extracromic flow. Purified GSH and GSSG were added to similar biological samples to confirm the chromatographic mobility (Sigma, St. Louis, MO) and to verify the identity of the observed peaks.

Statistics. The mean litter pup and fetal brain weight was calculated with the litter mean used for all statistical analysis. Percent reabsorptions were calculated by dividing the number of reabsorptions by the total number of fetuses (live + reabsorptions). Statistical analysis included ANOVA for continuous variables with Duncan’s New Multiple Range test, Mann-Whitney U test for nonparametric data, χ2 test for categorical variables, or Fisher’s Exact test where appropriate [Statview 4.5 (Abacus Concepts, Inc., CA)], with p < 0.05 considered significant.

Results

NAP + ADNF-9 Treatment Prevent Alcohol-Induced Fetal Death. Prenatal administration with NAP + ADNF-9 to alcohol-treated pregnant mice significantly increased the number of surviving fetuses in comparison with those given alcohol alone (Fig. 1). As previously described (Webster et al., 1980), alcohol treatment on gestational day 8 results in more than one-third of the fetuses dying in utero (Fig. 2). Pretreatment (30 min before alcohol administration) with NAP or the combination of NAP + ADNF-9 resulted in a number of surviving fetuses that were not different than that of control. Furthermore, these protective effects on fetal death were observed even when the peptides were administered 1 h after treatment with alcohol. NAP, alone or in combination with
ADNF-9, was required for the prevention of alcohol-induced fetal death. However, a 3-h post-treatment with the peptides did not result in a significant change in the number of surviving fetuses in comparison with the alcohol-treated group. In addition, treatment with NAP + ADNF-9 without alcohol cotreatment resulted in an incidence of fetal death that was not different from that of control (Fig. 2). Pretreatment with the neurotrophic peptide VIP did not prevent the alcohol-induced fetal death. The litter size (living + demises) was not different between the groups, with an average litter size of eight fetuses. The number of fetuses per treatment groups ranged between 140 and 400, and the litter mean was used for statistical analysis.

Combination of NAP + ADNF-9 Prevented Alcohol-Induced Fetal Growth Abnormalities. Both fetal brain and body weights were significantly smaller in the litters from alcohol-treated mice, compared with controls (Figs. 1 and 3). The effect of alcohol on body size shown in Fig. 1 depicts the maximal effect observed in the study. The summary of the effects on mean body weight for the various treatment groups is shown in Fig. 3. ANOVA analysis of these data indicated significant effects of alcohol treatment and significant improvements with peptide administration. With respect to changes in the mean body weight, pretreatment with NAP + ADNF-9 prevented the alcohol-induced fetal growth restriction and microcephaly. Fetal brain (A) and body (B) weights were obtained at E18. Pretreatment with NAP + ADNF-9 prevented the growth restrictions associated with alcohol. Post-treatment at 1 and 3 h with NAP + ADNF-9 prevented the microcephaly associated with alcohol. Comparisons are made to the alcohol group; overall ANOVA is \( p < 0.001 \). Post hoc Fisher's tests were performed, with the asterisked groups significantly different than alcohol. The mean from each litter was used for statistical analysis. Average litter size was 8 to 10 fetuses; thus, there were approximately 110 to 330 fetuses in each group. Sample sizes were control (33), alcohol (32), NAP + alcohol (24), NAP + NAP + alcohol (17), ADNF-9 + alcohol (11), VIP + alcohol (17), NAP + ADNF-9 + alcohol (19), NAP + ADNF-9 alone (19), NAP + ADNF-9 1 h post-alcohol (17), and NAP + ADNF-9 3-h post-alcohol (11).
ment with NAP + ADNF-9 prevented the growth restrictions associated with alcohol. Although neither peptide alone prevented alcohol-induced fetal growth abnormalities, the combination of the two was effective. This effect was not due to concentration, because pretreatment with a double dosage of NAP (40 μg) did not prevent the alcohol-induced growth restriction. This observation indicates that the combination of the two peptides was necessary to prevent alcohol-induced growth restrictions. Post-treatment with NAP + ADNF-9 (1 and 3 h) prevented the microcephaly, but not the growth restriction. Pretreatment with VIP did not prevent the alcohol-induced growth restrictions, and treatment with NAP + ADNF-9 without alcohol resulted in fetal weights that were not different than that of control (Fig. 3, A and B). Treatment with the peptides without alcohol resulted in body weights that were not different from those of controls. Average litter size was 8 to 10 fetuses, thus there were approximately 110 to 330 fetuses in each group.

Bioavailability of NAP. The biodistribution of [3H]NAP was studied after intraperitoneal injection of pregnant mice. These studies demonstrated that 68% of the radioactivity recovered in the embryo comigrated with intact NAP 30 min after administration (Fig. 4). Based on amount of radioactivity and the specific activity of the peptide, the calculated average accumulation of labeled peptide was approximately 0.2 pmol of NAP in the embryo, with an estimated concentration of 10 nM, well within the therapeutic range of NAP as determined from in vitro studies (Bassan et al., 1999). Analysis of the [3H]NAP associated with the embryos 60 min after injection indicated 39% comigrated with intact NAP.

Prevention of Oxidative Stress. Alcohol treatment resulted in a significant decline of GSH/GSSG to 50% of control (p < 0.005). Pretreatment with NAP + ADNF-9 (20 μg each) prevented the alcohol-induced decline in GSH/GSSG, with a ratio not different from that of the control (Fig. 5).

Discussion

Systemic administration of two peptides, NAPVSIPQ and ADNF-9, has been shown to prevent alcohol-induced fetal demise and growth restrictions in a model of FAS. The results of this study clearly indicated that treatment with NAP alone was effective in preventing alcohol-induced fetal death, whereas ADNF-9 given alone was not protective. Cotreatment with both peptides was necessary for the prevention of growth restriction produced by prenatal alcohol treatment. Increasing the concentration with a double dosage of NAP did not prevent the alcohol-induced growth restriction; whereas adding ADNF-9 produced a significant effect on growth restriction. Since the combinatorial peptide treatment was initiated 10 days before evaluation, these studies indicate a prolonged period of protection that was elicited by peptides. However, a period of effective intervention was apparent from the studies. Prevention of alcohol-induced fetal death occurred when the peptides were administered 1 h after alcohol administration. Thus, intervention in the severe toxicity induced by alcohol was effective even after a substantial delay in the onset of peptide treatment. However, delaying the peptide treatment to 3 h after alcohol treatment resulted in no demonstrable protection. These studies suggest that a period of irreversible damage occurs with alcohol toxicity, but significant long-term protection can be obtained with early treatment with NAP/ADNF-9.

Although the mechanism(s) of action of these peptides are not fully understood, the rationale for testing these drugs in the alcohol paradigm was due to their demonstrated effects in preventing oxidative damage and neuronal cell death in cell culture (Brenneman and Gozes, 1996; Bassan et al., 1999; Offen et al., 2000; Steingart et al., 2000). Although toxicity associated with ethanol is complex, oxidative damage due to the generation of free radicals and diminished reduced-glutathione appear to be important mechanistic components (Lieber, 1999; Mitchell et al., 1999). Peptide-mediated effects on oxidative stress have been identified through the inhibition of reactive oxygen species and mitochondrial peroxynitrite (Glazner et al., 1999a). Therefore, the potential of these peptides in preventing alcohol-induced damage appeared plausible. As an index of oxidative stress (Devi et al.,
1993), both reduced (GSH) and oxidized (GSSG) glutathione were measured in gestations (embryo + decidua) 8 h after treatment (Muscari et al., 1998). As previously shown (Devi et al., 1993), alcohol treatment significantly decreased the ratio of GSH/GSSG. Importantly, treatment with the peptides prevented the alcohol-induced changes in reduced oxidized glutathione levels in the embryo/deciduas and resulted in a ratio not different from that of control. The prevention of the alcohol-induced alteration of the GSH/GSSG ratio supports the conclusion that alcohol treatment does produce significant oxidative stress as assessed by this marker and that the peptides act as oxidative protectants that are mediated in part through glutathione. Other models support this conclusion: for example, treatment with antioxidative agents, such as vitamin E or beta-carotene, prevented alcohol-induced apoptotic cell death in cell culture (Mitchell et al., 1999). In related studies of NAP and ADNF-9-mediated protection from dopamine toxicity, a glutathione-related mechanism appears to be involved (Offen et al., 2000). Furthermore, toxicity associated with buthionine sulfoximine, a selective inhibitor of glutathione synthesis, was significantly attenuated with NAP treatment of neuroblastoma cells. Although the mechanism(s) of alcohol-induced damage and death are incompletely understood, it appears likely that a dysregulation of the oxidative state occurs after alcohol administration and that NAP/ADNF-9 treatment has an effect on an important regulator of oxidative homeostasis: glutathione.

Although the antioxidative properties of the peptides may play a major role in their mechanism of action, other studies have indicated other possible contributing actions. ADNF-9 has been shown to produce effects on transcriptional regulation that include an induction of NF-kB activation in hippocampal neurons (Glazner et al., 2000) and neurite extension via enhanced CAMP response element-binding protein phosphorylation in dorsal root ganglia cultures (White et al., 2000). These studies provide an operational framework to explain the observation that a short-term exposure to these peptides results in a long-term protection from toxicity, both in cell culture and in the FAS model described in the present paper. A transcriptional regulation of regulators of oxidation/reduction and/or apoptosis mediators may contribute to the observed long-term protection.

The nervous system is particularly vulnerable to ethanol toxicity at critical periods during development (Miller, 1995; Olney et al., 2000). Apoptotic death is produced by the addition of alcohol to neuronal cultures (Andrews et al., 1999). Recent studies have shown that in utero exposure to alcohol produced significant increases in apoptosis in the fetal brain (Ikonomidou et al., 2000). These effects were particularly evident in the hippocampus, thalamus, and cerebral cortex. Relevant to these events is previous experiments that demonstrated the prevention of apoptosis associated with electrical blockade in cerebral cortical cultures with ADNF, NAP, and ADNF-9 (Brenneman and Gozes, 1996; Brenneman et al., 1998; Bassan et al., 1999). Using a terminal deoxynucleotidyl transferase assay, ADNF produced inhibition of apopto-
sis in cerebral cortical cultures that was evident at femtomolar concentrations (Gozes et al., 1997). Furthermore, increased hsp60 expression in cortical neurons (Zamostiano et al., 1999) and inhibition of glutamate-induced calcium levels in hippocampal neurons (Guo et al., 1999) have been observed after ADNF-9 treatment. From these studies, it is likely that multiple mechanisms may contribute the protective properties of ADNF-9 in vivo. Treatment with NAP and ADNF-9 was more effective when administered in combination. This strongly suggests that NAP may work through complementary, but different, mechanisms.

Recent studies of NAP and ADNF-9 in other in vivo models provide additional points of comparison. In apolipoprotein E-deficient mice (Bassan et al., 1999) and in AP64A-treated animals (Gozes et al., 2000), NAP was more effective than ADNF-9 in providing long-term protection against loss of spatial memory. In mice exposed to closed-head injury, NAP attenuated the increases in tumor necrosis factor-alpha observed in the brain.

The discovery of protective peptides described herein has its basis in the neurotoxic and growth-promoting properties of VIP. Although VIP apparently has fundamental roles in the regulation of embryo/brain development (Gressens et al., 1993), the peptide itself is quickly degraded on systemic administration, making it a poor therapeutic agent. In contrast, several pharmacological properties of NAP and ADNF-9 form the basis of their unique therapeutic potential: potency at femtomolar concentrations and apparent stability in vivo, particularly in the case of NAP. Recovery of significant amounts of radioactive material comigrating with intact NAP in embryos 60 min after systemic administration attests to the stability of this peptide. These studies, in addition to providing a potential important agent for the study of alcohol-induced damage/death, suggest a promising approach to the treatment of human neurodegenerative disease that involves oxidative stress and/or dysregulation of apoptosis.

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