Maternal Alcohol Administration Suppresses Expression of Nitric Oxide Synthase in the Hippocampus of Offspring Rats

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Abstract. Maternal alcohol consumption during pregnancy can produce teratogenic effects and has a detrimental effect on the development of the fetus. In this study, the dose-dependent effect of maternal alcohol administration on the expression of nitric oxide synthase (NOS) in the hippocampus of the offspring rats was investigated. From the present result, it was shown that expression of NOS is decreased following treatment with maternal alcohol in a dose-dependent fashion. The present results suggest that suppression of NOS expression in the hippocampus of offspring rats with maternal alcohol mediates the associated developmental retardation and/or anomalies.

Keywords: maternal alcohol, nitric oxide synthase, hippocampus

Excessive maternal alcohol consumption during pregnancy can produce teratogenic effects and has a detrimental effect on the development of the fetus. Prenatal alcohol exposure induces brain injury in offspring that can manifest, in part, as life-long learning and cognitive deficits. Among the brain regions shown to be affected adversely by prenatal exposure to alcohol in animal models, the hippocampus is a selectively vulnerable structure to prenatal ethanol exposure (1). It is generally accepted that prenatal ethanol exposure can alter hippocampal synaptic plasticity and can adversely affect hippocampal-dependent learning, as well as cause other mental defects. Previous studies using the guinea pig have demonstrated that chronic prenatal ethanol exposure results in loss of hippocampal CA1 pyramidal cells in young postnatal offspring (2) and decreases synaptic plasticity in the hippocampus of adult offspring (3).

Nitric oxide (NO) acts as a neurotransmitter and a biological messenger molecule in the brain and other mammalian tissues. It has been shown that alcohol inhibits NO production in vivo, and thus it may be suggested that NO is of relevance in the pathogenesis of alcohol-induced brain damage (4). Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is a histochemical marker specific for nitric oxide synthase (NOS) in the central nervous system (CNS). Neurons containing NADPH-d have been reported to be relatively resistant to various toxic insults and neurodegenerative disorders (5). In the present study, the dose-dependence of the effect of maternal alcohol administration on NOS expression in the hippocampus of offspring rats was investigated.

Adult female Sprague-Dawley rats weighing 220 ± 10 g were used in the experiment. A total of twenty female rats were mated in pairs with 20 adult male rats weighing 300 ± 10 g for 48 h. After mating, the female rats were housed individually in a plastic home cage for 2 weeks at a controlled temperature (20 ± 2°C) and under light-dark cycles consisting of 12 h of light and 12 h of darkness (light on from 07:00 h to 19:00 h), with food and water made available ad libitum. The experimental procedures were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society and the Korean Academy of Medical Sciences.

After confirmation of pregnancy, the female rats were randomly divided into four groups: the control group,
the 0.5 g/kg maternal alcohol administration group, the 1 g/kg maternal alcohol administration group, and the 2 g/kg maternal alcohol administration group (n = 4 in each group). Starting on the 15th day of pregnancy, rats of the control group were injected subcutaneously with saline once a day for 7 consecutive days, and animals of the maternal alcohol administration groups were injected subcutaneously with alcohol at the respective doses once a day over the same period of time. After birth, the offspring of each female rat were left undisturbed together with the mother; they (n = 10 in each group) were sacrificed 3 weeks after birth.

To begin the sacrificial process, animals were first fully anesthetized with Zoletil 50® (10 mg/kg, i.p.; Vibac, Carros, France), transcardially perfused with 50 mM phosphate-buffered saline (PBS), and then fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). The brains were then removed, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40-µm thickness were made with a freezing microtome (Leica, Nussloch, Germany). Sections were then stained for NADPH-d activity according to a previously described protocol (6). Eight sections on average were selected from the region spanning from Bregma -3.30 mm to -4.16 mm in each brain. In brief, free-floating sections were incubated at 37°C for 60 min in 0.1 M PB containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium, and 0.1 mg/ml β-NADPH. The sections were then washed three times with PBS and mounted onto gelatin-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted using Permount® (Schleicher & Schuell GmbH, Dassel, Germany). Mouse nNOS antibody (1:500; Santa Cruz Biotech, Santa Cruz, CA, USA) was used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse antibody for nNOS was used as secondary antibodies. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech GmbH, Freiburg, Germany). Statistical differences were determined using the independent t-test, and results were expressed as the mean ± S.E.M. Differences were considered significant for P<0.05.

The numbers of NADPH-d-positive cells in the each region of the hippocampus from the experimental groups are summarized in Table 1. Expression of NOS in CA1 and the dentate gyrus of offspring rats of the maternal alcohol treatment group were significantly decreased compared to the control group in a dose-dependent manner. In contrast, NADPH-d-positive neuron expression was not affected or slightly decreased with statistically insignificance in the CA2-3 regions of the hippocampus.

In RT-PCR analysis, with respect to the mRNA level of nNOS in the control group (control value, 1.00), the level of nNOS mRNA in the hippocampus was found to be markedly reduced in all of the maternal alcohol administration groups, to 0.72 ± 0.03 in the 0.5 g/kg maternal alcohol administration group, to 0.54 ± 0.03 in the 1 g/kg maternal alcohol administration group, and to 0.37 ± 0.03 in the 2 g/kg maternal alcohol administration group (Fig. 1). In Western blot analysis, nNOS protein was observed mainly in a band of approximately 155 kDa. Expression of nNOS protein in the hippocampus of each group (per section) are summarized in Table 1. Expression of nNOS in CA1 and the dentate gyrus of offspring rats of the maternal alcohol treatment group were significantly decreased compared to the control group in a dose-dependent manner. In contrast, NADPH-d-positive neuron expression was not affected or slightly decreased with statistically insignificance in the CA2-3 regions of the hippocampus.

Table 1. Number of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)-positive cells in the hippocampus of each group (per section)

<table>
<thead>
<tr>
<th>Group</th>
<th>CA1</th>
<th>CA2-3</th>
<th>Dentate gyrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82.36 ± 2.78</td>
<td>30.56 ± 1.57</td>
<td>78.56 ± 2.97</td>
</tr>
<tr>
<td>0.5 g/kg maternal alcohol-treated</td>
<td>75.12 ± 3.52</td>
<td>29.12 ± 2.02</td>
<td>72.36 ± 2.26</td>
</tr>
<tr>
<td>1 g/kg maternal alcohol-treated</td>
<td>69.32 ± 3.20*</td>
<td>25.52 ± 1.79</td>
<td>61.24 ± 2.80*</td>
</tr>
<tr>
<td>2 g/kg maternal alcohol-treated</td>
<td>55.08 ± 15.08*</td>
<td>25.24 ± 2.02</td>
<td>57.36 ± 3.58*</td>
</tr>
</tbody>
</table>

Values each represent a mean ± S.E.M. Statistical differences were determined using independent t-test. *P<0.05, compared to the control group. Differences were considered significant for P<0.05.
Maternal alcohol consumption during pregnancy is known to cause anomalies in fetal development, of which fetal alcohol syndrome is one manifestation. The teratogenic effects of alcohol on the fetal CNS can result in intellectual, behavioral, and/or motor dysfunctions in the offspring. One of the target sites of the teratogenicity of alcohol in the CNS is the hippocampus that is involved in learning and memory (8). Prenatal alcohol exposure restricts hippocampal growth in the near-term fetus (1) and decreases the number of CA1 pyramidal neurons in the adult (8). In addition, alcohol impairs the acquisition of hippocampus-driven spatial memory in adolescents as well as in adults (9). Dose-response studies have shown that alcohol potently inhibits synaptic activity in hippocampal slices of juvenile rats (9, 10). As put forth in these studies, substantial evidence indicates that administration of alcohol in large amounts over a short time span disrupts cellular activity in the hippocampus and that the effect of alcohol on hippocampal cellular activity is likely to contribute to its deleterious effect on learning and memory.

In the CNS, NO has been shown to play a role in neurotransmitter release from brain and synaptosomes, as well as to play a role in brain development (11). In particular, nNOS, one of the several isoforms of NOS, has been suggested to modulate signal transmission and synaptic plasticity in neuronal cells (12). It has been suggested that prenatal alcohol exposure can affect NOS expression in the hippocampus. Kimura et al. (13)
suggested that chronic maternal administration of alcohol throughout gestation suppresses hippocampal NOS activity in the near-term (gestational day 62) fetal guinea pig by about one-third, but does not affect NOS activity in the postnatal offspring at postnatal day 12, 22, or 62 (14). It has been demonstrated, using the same ethanol regimen as in the aforementioned study, that chronic prenatal ethanol exposure produces a 25% loss of CA1 pyramidal cells in the postnatal day 12 offspring and decreased hippocampal weight, but did not affect hippocampal NOS activity in the postnatal guinea pig (14). On the other hand, Dizon et al. (15) reported that antenatal alcohol exposure increased hippocampal NOS levels in the offspring (gestational day 55–60) of guinea pigs. These results showed that maternal alcohol administration increased nNOS protein level expression and both Ca\(^{2+}\)/CaM-dependent and -independent NOS activity within the guinea-pig brain. Controversy continues to this date regarding the effects of prenatal alcohol exposure on NOS expression in the hippocampus of offspring. From the results of the present study, the expression of NOS in the hippocampus of offspring rats was decreased by maternal alcohol administration during pregnancy in a dose-dependent manner. These results suggest that maternal alcohol-induced suppression of NOS expression in the hippocampus of offspring rats may be an underlying mechanism of alcohol-induced developmental disorders in the offspring following maternal alcohol administration.

References