OBSTETRICS

The role of NADPH oxidase in a mouse model of fetal alcohol syndrome

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OBJECTIVE: Fetal alcohol syndrome (FAS) is the most common cause of nongenetic mental retardation. Oxidative stress is one of the purported mechanisms. Nicotinamide adenine dinucleotide phosphate oxidase (NOX) is an enzyme involved in the production of reactive oxygen species. Our objective was to evaluate NOX in the fetal brain of a well-validated mouse model of FAS.

STUDY DESIGN: Timed, pregnant C57BL/6J mice were injected intraperitoneally with 0.03 mL/g of either 25% ethyl alcohol or saline. Fetal brain, liver, and placenta were harvested on gestational day 18. The unit of analysis was the litter; tissue from 6-8 litters in the alcohol and control group was isolated. Evaluation of messenger ribonucleic acid (mRNA) expression of NOX subunits (DUOX1, DUOX2, NOX1, NOX2, NOX3, NOX4, NOXA1, NOXO1, RAC1, p22phox, and p67phox) was performed using quantitative real-time polymerase chain reaction; alcohol vs placebo groups were compared using a Student *t* test or a Mann-Whitney test ($P < .05$).

RESULTS: Alcohol exposed fetal brains showed significant up-regulation in subunits DUOX2 (1.61 ± 0.28 vs 0.84 ± 0.09; $P = .03$), NOXA1 (1.75 ± 0.27 vs 1.09 ± 0.06; $P = .04$), and NOXO1 (1.59 ± 0.10 vs 1.28 ± 0.05; $P = .02$). Differences in mRNA expression in the placenta were not significant; p67phox was significantly up-regulated in alcohol-exposed livers.

CONCLUSION: Various NOX subunits are up-regulated in fetal brains exposed to alcohol. This effect was not observed in the fetal liver or placenta. Given the available evidence, the NOX system may be involved in the causation of FAS through the generation of reactive oxygen species and may be a potential target for preventative treatment in FAS.

Key words: fetal alcohol syndrome, nicotinamide adenine dinucleotide phosphate oxidase, reactive oxygen species

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Despite a 1981 Surgeon General report warning against the use of alcohol in pregnancy in the United States, up to 50% of child-bearing aged women drink alcohol and up to 20% continue to consume alcohol after finding out they are pregnant; 1 in 25 pregnant women admit to binge drinking.[1,2] A study in 5 states in the United States from 1995 to 1997 revealed a fetal alcohol syndrome (FAS) prevalence rate from 0.3 to 1.5 cases per 1000 live births.[3] FAS is the most common cause of mental retardation not caused by genetics.[4]

Alcohol crosses the placenta yet the exact quantity of alcohol that is deleterious to a growing fetus remains unknown. Binge drinking is associated with a more negative impact on pregnancy than small amounts of alcohol consumption.[5,6] Moderate alcohol consumption in early gestation has not been shown to affect the intelligence quotient of offspring at 8 years of age.[7] However, in a separate study, women who consumed more than 70 g of alcohol in a week's time (or binge drank 1-2 times per week) during the first trimester had an increased risk of alcohol-related birth defects.[8]

The brain accounts for 20% of total body oxygen consumption.[9] Oxygen consumption causes the generation of free radicals, and these may be increased in response to alcohol exposure. The antioxidative system functions to prevent cellular damage produced by free radicals. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are members of the antioxidant system and are present in the cerebral cortex, cerebellum, and hypothalamus.[10] We have previously shown that maternal alcohol exposure causes a decrease in messenger ribonucleic acid (mRNA) expression of SOD, GPx, and CAT in the fetal brain.[11]

Nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase (NOX) has been found to play a significant role in ethanol induced oxidative stress and has been identified as a source of reactive oxidative species (ROS) in mouse embryos exposed to ethanol.[12] The NOX system consists of an intricate web of mechanisms for activation

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that ensure the ROS created are regulated in quantity and duration of function.[13] NOX1 through NOX5 each require different complexes and regulators to function[14] including catalytic (DUOX1 and DUOX2) and regulatory (p22phox, p47phox, p67phox, NOXA1, and NOXO1) subunits (Figure 1). For example, NOX1 through NOX4 require an intramembranous activator, or maturation factor, called p22phox.[13] Our goal was to determine whether FAS is associated with the up-regulation of NOX in the fetal brain.

Materials and Methods

Animal care and dosing

The study protocol and all related procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (Galveston, TX). Pregnant C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were received, and vaginal plug detection was considered gestational day 0. The mice were maintained in the animal care facility at the University of Texas Medical Branch, housed separately in temperature- and humidity-controlled quarters with constant 12-hour light/12-hour dark cycles, and provided with food and water ad libitum. A well-characterized mouse model of FAS was used.[15]

On gestational day 8, pregnant mice in the alcohol group received an intraperitoneal injection of 25% ethyl alcohol (0.03 mL/g per body weight), whereas those in the control group received an equivalent weight-based dose of saline. On gestational day 18, the pregnant mice were euthanized with carbon dioxide. The placenta, fetal brain, and portion of the fetal liver were immediately flash frozen in liquid nitrogen and stored at −80°C. A total of 8 mice received alcohol and 7 received saline, yielding 53 alcohol- and 50 saline-exposed pups.

RNA isolation and reverse-transcriptase reaction

Tissue samples from each fetus (brain, placenta, and liver) were analyzed individually. Samples were homogenized using Bullet Blender from Next Advance (Averill Park, NY). To adequately represent each litter, 2-3 pup samples per litter were chosen at random to achieve a sample size of 20 for the alcohol group and 20 for the saline group. Total ribonucleic acid (RNA) was isolated using Trizol and a Zymo RNA isolation kit (Ambion, Austin, TX, and Zymo Research Corporation, Irvine, CA) according to the manufacturer's instructions. Quantification of RNA was performed by measuring the absorbance of RNA sample solutions at 260 nm. For the reverse transcription reaction, we used a high-capacity complementary deoxyribonucleic acid reverse transcription kit (Applied Biosystems, Foster City, CA) with the manufacturer manual (25°C for 10 minutes → 37°C for 120 minutes → 85°C for 5 minutes → 4°C for infinity).

Quantitative real-time polymerase chain reaction

SYBR green polymerase chain reaction (PCR) master mix (2 times) (Applied Biosystems, Warrington, UK) was used according to the manufacturer's instructions for quantitative real-time PCR performed in a 7500 Fast real-time PCR system (Applied Biosystems). Mouse-specific TaqMan primers (Applied Biosystems) were used (Table 1). Gene expression was calculated as the mRNA of the targeted gene relative to the glyceraldehyde-3-phosphate dehydrogenase mRNA levels in each specific sample (relative unit). Each reaction was carried out in duplicate. The relative quantification was determined using 7500 software version 2.06 (Applied Biosystems).

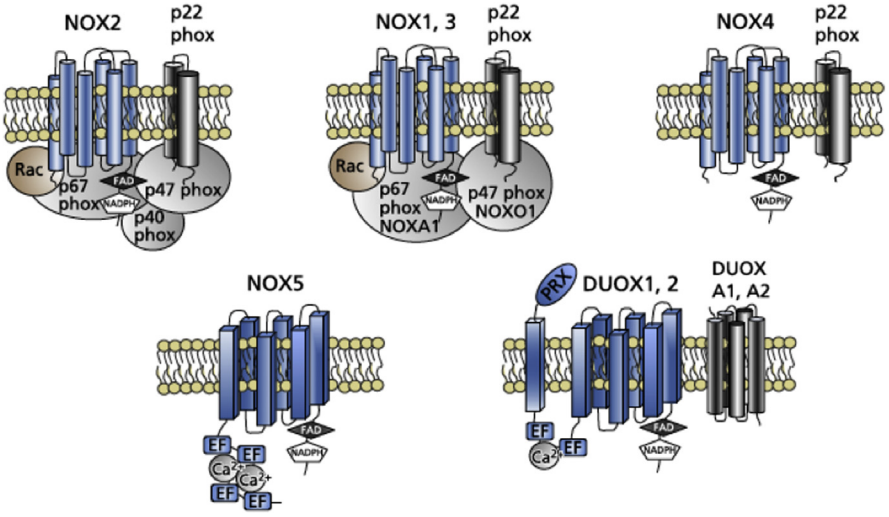
Data analysis

The unit of analysis was the litter. Statistical analysis was performed using GraphPad Software, Inc (version 5.04; La Jolla, CA). Data are expressed as mean ± SEM. A Shapiro-Wilk test to check for normality was performed, and then a Student *t* test or Mann-Whitney test was used accordingly. A 2-tailed value of $P < .05$ was considered statistically significant.

Results

The mrna expression of DUOX2 (1.61 ± 0.28 vs 0.84 ± 0.09; $P = .03$), NOXA1 (1.75 ± 0.27 vs 1.09 ± 0.06; $P = .04$), and NOXO1 (1.59 ± 0.10 vs 1.28 ± 0.05; $P = .02$) was found to be significantly increased in the brains of litters born to dams exposed to alcohol compared with the control group (Figure 2). Increased mRNA expression was noted

FIGURE 1
Illustrated diagrams of NADPH



EF, EF-hands; *FAD*, flavin adenine dinucleotide; *NADPH*, nicotinamide adenine dinucleotide phosphate; *PRX*, peroxidase-like domain. Reproduced, with permission, from Katsuyama et al.[14]