Vascular function in alcohol-treated pregnant and nonpregnant mice

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Cook, Jocelynn L., Yunlong Zhang, and Sandra T. Davidge. Vascular function in alcohol-treated pregnant and nonpregnant mice. Am J Physiol Regulatory Integrative Comp Physiol 281: R1449–R1455, 2001.—The effect of alcohol on maternal vascular adaptations to pregnancy is unknown. This study was designed to determine the effect of alcohol consumption on nitric oxide-mediated vascular function in mice during pregnancy. Female pregnant or nonpregnant C57BL/6J mice were fed a control diet or a liquid diet of 25% ethanol-derived calories for 13 days (from gestational days 6–18). Phenylephrine vasoconstriction was blunted in pregnancy compared with the nonpregnant state due to enhanced nitric oxide modulation, which was impaired by ethanol exposure. Although the EC50 and maximal responses to methacholine were not different in nonpregnant vs. pregnant mice, the nitric oxide component to methacholine-induced vasorelaxation was greater in the pregnant mice. Interestingly, alcohol affected only the pregnant animals in their response to methacholine. These data indicate that alcohol reduced the nitric oxide modulation of vascular response, which was more pronounced during pregnancy. These studies provide novel information regarding the effects of alcohol on the maternal vascular system during pregnancy and thereby contribute to further understanding of the adverse effects associated with prenatal alcohol exposure.

nitric oxide; mouse

THE ASSOCIATION BETWEEN ALCOHOL consumption and cardiovascular morbidity and mortality has been described as an inverse J-shaped relationship (40). For example, small to moderate amounts of alcohol may be cardioprotective (1), whereas chronic alcohol abuse has negative effects on the cardiovascular system (39). Specifically, excessive alcohol use may raise blood pressure, damage the myocardium, and precipitate arrhythmias (8).

Most studies have investigated the relationship between alcohol use and cardiovascular function in males (3, 29). However, it is also important to understand the effects of alcohol consumption on cardiovascular function in females. The relationship between alcohol consumption during pregnancy and maternal cardiovascular function deserves special consideration, because it has long been recognized that alcohol consumption during pregnancy is associated with serious maternal and fetal complications (21, 33). Previous studies have focused on the effects of alcohol on vascular tissues of fetal origin (4, 5, 13, 35, 37), but, surprisingly, there is a paucity of literature about the effect of alcohol on the maternal cardiovascular system during pregnancy.

The maternal cardiovascular system plays a unique and important role in pregnancy. During pregnancy, there is a 45% increase in plasma volume (9), and the cardiovascular system efficiently adapts to accommodate these changes. There is a fall in peripheral resistance, an increase in cardiac output (9), a substantial reduction in pressor responsiveness to exogenously administered vasoconstrictors (42), and a reduction in vascular contractile ability (31). Pregnancy-associated diseases that compromise both maternal and fetal health, including pregnancy-induced hypertension, gestational diabetes, and eclampsia/preeclampsia, occur when the maternal cardiovascular system does not adapt to the conditions of pregnancy (14, 32).

The vascular adaptations associated with pregnancy are mediated in part by nitric oxide (NO). Studies in rats suggest that the vasodilatory actions of NO allow peripheral vessels to accommodate the increases in blood flow and the high metabolic requirements of the maternal and feto-placental tissues (7, 30). Alcohol has been shown to affect NO production in vivo (16, 24) and in vitro (12, 17) and affects vascular reactivity (3, 29), thus it is possible that NO modulation of the vascular adaptations associated with pregnancy are altered in response to alcohol consumption. Therefore, we hypothesized that alcohol may affect NO-mediated cardiovascular adaptations of pregnancy.

METHODS

Animals and Breeding

Female C57BL/6J mice (10–12 wk of age, Jackson Laboratories, Bar Harbor, ME) were used in the experiment. Mice were housed in groups of four in polypropylene cages in a temperature- and humidity-controlled environment. Animal housing rooms were on a 12:12-h light-dark cycle (lights on at 0700 h and off at 2300 h) in a clean cage system with bedding changed at least once per week. All mice were allowed ad libitum access to food and water and were handled at least once daily to minimize any stress associated with immobilization and injection procedures. The room temperature was maintained at 22°C ± 1°C, and the relative humidity was 55 ± 10% throughout the experiment.

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Dams in the alcohol groups were given free access to a liquid diet (chocolate Sustacal, Mead Johnson) as their sole source of food and fluid. The diet was fortified with vitamins and minerals (Salt Mixture XIV), both from ICN Nutritional Chemicals (Aurora, OH), as described in Diet Preparation. Pregnant dams received the diet from GD 6–18, and nonpregnant animals received the diet for 13 days. Diets were administered in graduated conical tubes with spouts. Fresh diets were given between 1500 and 1600 daily, ~2 h before the beginning of the dark cycle. This liquid diet is commonly used to study effects of chronic alcohol consumption during pregnancy (34). The control groups (pregnant and nonpregnant) were allowed free access to laboratory chow and water. Pilot studies with the liquid diet made isocaloric to the EtOH diet by addition of sucrose indicate that there are no differences between sucrose-treated and ad libitum laboratory chow controls in terms of weight gain during pregnancy (78.93 g ± 5.83 g vs. 77.82 g ± 10.55 g, P > 0.05), number of pups per litter (6.5 ± 0.46 vs. 6.25 ± 1.60, P > 0.05), mean litter weight (1.04 g ± 0.06 vs. 1.09 g ± 0.035 g, P > 0.05), mean placental weight (0.1 g ± 0.01 g vs. 0.1 g ± 0.006 g, P > 0.05), and mean fetal brain weight (0.08 g ± 0.01 g vs. 0.08 g ± 0.007 g, P > 0.05). Thus the observed effects were not due to nutritional or diet variables, and the ad libitum laboratory group was used as the control group for these studies. Two separate alcohol-treated groups (pregnant and nonpregnant) were used to determine time-dependent changes in blood alcohol levels. Blood samples were taken from the retro-orbital sinus of the eye at 1800 and 2400 on treatment day 12 and at 0600 and 0900 on treatment day 13 and analyzed as described in Blood Alcohol Measurement. The animal protocol was examined by the University of Alberta Animal Welfare Committee and found to be in compliance with the guideline issued by the Canada Council on Animal Care.

Diet Preparation

The alcohol diet consisted of 25% ethanol-derived calories (EDC). The EDC liquid diet was prepared by adding 22.0 ml of 95% alcohol to 335 ml of chocolate Sustacal. This mixture was brought up to 480 ml with water. ICN vitamins (1.44 g) and minerals (1.2 g) were added to the diet mixture.

Vessel Preparation and Equipment

Dams were weighed daily and were killed after 13 days of alcohol treatment (GD 18). The normal gestational length of this mouse strain is 19 days. Pups were collected by cesarean section from pregnant animals, and litter body weights and brain weights were recorded. Trunk blood was collected from the dams at the time of death and used for blood alcohol measurement (see Blood Alcohol Measurement).

Because pregnancy increases mesenteric blood flow by 75% (23) and we have previously shown pregnancy-induced alterations in isolated mesenteric artery in the rat (11), we assessed the effects of pregnancy and alcohol on resistance-sized mesenteric arteries in the mouse. A section of the mesentery was rapidly removed from the experimental animals and placed in ice-cold HEPES-buffered physiological saline solution (HEPES-PSS; in mmol/l: 142 NaCl, 4.7 KCl, 1.17 MgSO4, 1.56 CaCl2, 1.18 KH2PO4, 10 HEPES, and 5.5 glucose). Mesenteric arteries (2nd order branches) were carefully dissected from adipose tissue and transferred to a dual chamber arteriograph (Living Systems Instrumentation, Burlington, VT). The proximal end of the artery was tied to a glass cannula of the arteriograph with silk thread, and the artery was gently flushed with HEPES-PSS buffer to remove residual blood with a servo pump. Then the distal end of the artery was mounted to a second glass cannula, and intraluminal pressure was gradually increased to 50 mmHg to parallel the in vivo arterial pressure. All arterial measurements, including inner diameter and wall thickness, were collected by a video camera mounted on the microscope, a dimension analyzer (Living Systems Instrumentation, Burlington, VT), and a monitor.

Experimental Protocol

Arteries were equilibrated in warm (37°C) HEPES-PSS buffer for 30 min at an intraluminal pressure of 50 mmHg. The arteries were prestretched by increasing the intraluminal pressure from 50 to 75 mmHg and immediately returning it to 50 mmHg. This pressure was maintained throughout the experiment period and arteries were allowed to equilibrate for 30 min after the period of prestretch.

Two experimental protocols were used: 1) phenylephrine-induced vasoconstriction and 2) endothelium-dependent vasodilation to methacholine. Cumulative doses of phenylephrine (0.1–10 μmol/l) were conducted in the absence or presence of a nitric oxide synthase (NOS) inhibitor, Nω-monomethyl-L-arginine (L-NMMA, 250 μmol/l). Cumulative doses of methacholine (1 nmol/l–1 μmol/l) were applied to arteries that were preconstricted with phenylephrine to their EC50 in the absence or presence of L-NMMA. The reproducibility of repeating curves was determined in preliminary experiments.

Blood Alcohol Measurement

A blood sample (~50 μl) was collected in a heparinized capillary tube from the orbital sinus of each animal 60 min postintubation. Samples (10 μl) of blood were diluted 1:50 (wt/vol) after collection with 3.4% perchloric acid, vortexed, and centrifuged at 2,000 rpm for 15 min. Aliquots of the supernatant (10 μl) were assayed in duplicate by a spectrophotometric assay. In this assay, ethanol is converted to acetaldehyde by alcohol dehydrogenase (Sigma, St. Louis, MO), and NAD (Sigma) is stoichiometrically reduced to NADH. This reduction is maximally detected by ultraviolet spectrophotometry (340 nm).

Statistical Analyses

Data are summarized as the means ± SE. The data from the dose-response curves were fitted to the Hill equation, from which a straight line was generated by linear least regression analysis. EC50 was determined from this line and expressed as the geometric mean ± SE. Two-way ANOVA was used to analyze EC50 and maximal response data. When only two groups were compared (see Table 1), the unpaired Student’s t-test was used. Data were considered significantly different at values with P < 0.05.
RESULTS

Animal Model

Results of alcohol treatment are illustrated in Table 1. Although maternal weight gain and the average number of pups per litter were not affected by alcohol, pups born to alcohol-treated dams were smaller in body weight and had smaller brains than the control pups. As a consequence, the brain weight/body weight ratio was significantly lower than the ratio of control animals. As expected, blood alcohol levels from untreated dams were negligible. Baseline arterial diameters were similar among the groups.

Vasoconstrictor Response to Phenylephrine

The maximal responses to phenylephrine were not different among the groups. Concentration response curves to phenylephrine are illustrated in Fig. 1, and data are summarized as EC50 values in Fig. 2.

Effect of pregnancy. Pregnancy significantly reduced sensitivity of mesenteric arteries to phenylephrine (P = 0.017) as evidenced by the increased EC50 for phenylephrine (Fig. 2A). After incubation with L-NMMA, the EC50 of phenylephrine was significantly decreased in both pregnant (P < 0.001) and nonpregnant mice (P < 0.05). Because differences between EC50 values from vessels of pregnant and nonpregnant mice were no longer evident after NO inhibition by L-NMMA, these data suggest that the NO pathway contributes to the decreased sensitivity to phenylephrine-induced vasoconstriction in pregnant mice.

Effect of alcohol. Alcohol consumption by nonpregnant mice significantly increased vessel sensitivity to phenylephrine-induced vasoconstriction (P = 0.048; Fig. 2B). When NO production was blocked by L-NMMA, the sensitivity of the vessels to phenylephrine-induced vasoconstriction was increased in both nonpregnant groups. The increased sensitivity to phenylephrine and the response to NO inhibition were also seen in pregnant dams treated with alcohol compared with the pregnant control dams (P < 0.001; Fig. 2C). However, the magnitude of the change in sensitivity in vessels from alcohol-treated dams was less than the magnitude of the shift in control mice (Fig. 2, B and C). Also, vascular responses of vessels from alcohol-treated pregnant mice were not significantly different (P > 0.05) compared with responses from nonpregnant control mice, so alcohol treatment appeared to abolish pregnancy-associated NO modulation of vascular function. Overall, these data suggest that the NO component to phenylephrine-induced vasoconstriction is blunted in response to alcohol consumption.

Arterial Response to Methacholine

The EC50 responses for methacholine were not different between nonpregnant and pregnant mice, and
alcohol treatment affected only the maximal response to methacholine in the pregnant animals. Concentration response curves for methacholine-induced vasorelaxation are illustrated in Fig. 3, whereas data assessing changes in maximal responses to methacholine are depicted by Fig. 4.

**Effect of pregnancy.** Figure 4A illustrates that pregnancy did not affect the vasodilatory response to methacholine in untreated animals ($P = 0.825$). However, as shown in the control groups in Figs. 4, B and C, the data do suggest that the NO component to methacholine-induced vasorelaxation is greater in pregnancy, as shown by the significantly greater shift in response to NO inhibition by L-NMMA in pregnant animals compared with nonpregnant animals ($54.3 \pm 2.9\%$ vs. $22.8 \pm 2.8\%, P < 0.05$). Concomitant exposure to L-NMMA and the prostaglandin synthesis inhibitor meclofenate did not alter vascular function (data not shown). Together, these data indicate that vasodilators other than NO and prostaglandins predominate in the methacholine-induced vasorelaxation in nonpregnant vessels, whereas there is an enhanced NO component to the methacholine relaxation response in the pregnant animals.

**Effect of alcohol.** Figure 4B illustrates that maximal vascular responses to methacholine and the effect of NO inhibition were similar in alcohol-treated and control nonpregnant mice. Interestingly, alcohol affected only the pregnant animals in their response to methacholine. As shown in Fig. 4C, alcohol consumption during pregnancy reduces the maximal response to methacholine, and NO inhibition had less of an effect on vascular responsiveness to methacholine in the alcohol-treated mice. This is illustrated by the greater shift in sensitivity to methacholine with NO inhibition.

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![Fig. 2. Vascular response (EC$_{50}$) to phenylephrine in the presence or absence of (w/o) $N^\gamma$-monomethyl-L-arginine (L-NMMA), a nitric oxide synthase (NOS) inhibitor. A: the effect of pregnancy on vascular response to phenylephrine. B and C: the effect of alcohol consumption on vascular response to phenylephrine in nonpregnant and pregnant mice, respectively. Values are expressed as means ± SE. *$P < 0.05$ vs. control; †$P < 0.05$ vs. L-NMMA.](http://ajpregu.physiology.org/)

![Fig. 3. Concentration response curves to methacholine. The effect of chronic alcohol consumption on vascular responses to methacholine in nonpregnant (A) and in pregnant (B) mice. Values are expressed as means ± SE.](http://ajpregu.physiology.org/)
in the normal pregnant dams compared with the smaller shift in sensitivity in the alcohol-treated pregnant mice (54.3 ± 2.9% vs. 23.2 ± 8.4%, P < 0.05). These data imply that alcohol reduced the NO component of the relaxation response to methacholine.

DISCUSSION

This study was designed to determine the effects of alcohol consumption on maternal vascular adaptations to pregnancy. In addition, we conducted the studies using mice, because they have been used extensively to investigate mechanisms underlying effects of alcohol on fetal growth and development (36, 38). However, very little has been reported regarding the mechanisms of vascular adaptations of pregnancy in mice. Data from our study confirm the hypothesis that NO mediates some of the vascular adaptations associated with pregnancy in the mouse.

Mesenteric arteries from pregnant mice were less sensitive to the vasoconstrictive actions of phenylephrine. However, inhibition of NO by L-NMMA abolished the difference in sensitivity between pregnant and nonpregnant animals, suggesting that the blunted vasoconstrictor response to phenylephrine associated with pregnancy was mainly due to NO. Similar findings have been found in rat resistance-sized mesenteric arteries, where flow-mediated vasodilation was greater (26) and adrenergic-mediated vasoconstriction was reduced (11) in vessels from pregnant animals compared with the nonpregnant control group.

In the systemic circulation, the principal endothelium-dependent vasodilators are NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF). In our study, it is interesting that the overall relaxation to the endothelial-dependent relaxing agent methacholine was not different in pregnant mice compared with nonpregnant mice; however, the relative contribution of the vasodilator components were different. In the arteries of the pregnant mice, NO-mediated relaxation to methacholine was greater compared with the nonpregnant animals. Inhibition of the prostaglandin pathway did not alter relaxation responses in either group of mice. These data indicate that an additional vasorelaxant, other than prostacyclin or NO, predominated more in the nonpregnant mice compared with the pregnant animals. EDHF is a likely contributor to the vasodilator response in the nonpregnant animals (6). However, our data indicate that during pregnancy there is a shift to a predominant NO-dependent relaxation.

It is the predominance of the NO modulation of vascular tone that may increase the susceptibility of pregnant animals to the effects of alcohol. Although alcohol is commonly known as a peripheral vasodilator (2), it has been firmly established that regular alcohol use is associated with hypertension (25). Mechanisms underlying alcohol-induced hypertension remain elusive, but possible mechanisms may include an increase in sympathetic activity, an increase in intracellular calcium levels with a subsequent increase in vascular reactivity, and inhibition of endothelium-dependent vasorelaxation (18). A recent study lends light to the latter hypothesis, because endothelium-dependent vasodilation of brachial arteries of chronic alcoholic men was found to be impaired compared with the responses of nonalcoholics (29). Animal studies also support these findings. It has been found that chronic alcohol consumption by rats inhibits endothelium-dependent relaxation responses to acetylcholine (19). In a similar study, acute administration of ethanol produced con-

Fig. 4. Vascular response (maximal relaxation) to methacholine in the presence or absence of L-NMMA, a NOS inhibitor. A: the effect of pregnancy on the maximal relaxation response to methacholine. B and C: the effect of alcohol consumption on maximal relaxation response to methacholine in nonpregnant and pregnant mice, respectively. Values are expressed as means ± SE. #P < 0.05 vs. control; *P < 0.05 vs. L-NMMA.
centration-related vasoconstriction of arterioles and venules in skeletal muscle microvasculature of rats (3). Finally, in vitro assessment of vascular contractility of mesenteric resistance vessels of male rats fed alcohol for 18 wk suggested that ethanol consumption significantly enhanced vascular contractility to norepinephrine (20).

Our findings suggest that alcohol consumption blunts NO-mediated vasodilation that may be important to altered vascular function, especially during pregnancy. As discussed earlier, relaxation responses to methacholine in nonpregnant mice were due to a vasorelaxant (likely EDHF) that was not NO or prostacyclin. Alcohol did not alter this relaxation response in nonpregnant animals. However, alcohol did have an impact on vascular function in the pregnant animals. There was a large shift in vessel sensitivity to phenylephrine and methacholine in control pregnant animals after NO inhibition; however, the shift in the response in vessels from alcohol-treated pregnant dams was greatly attenuated. These data indicate a loss of NO modulation of vascular function in the pregnant animals. Whether NO synthesis or bioavailability is decreased or vascular smooth muscle sensitivity is altered by alcohol exposure is not yet known. Detailed mechanistic studies are difficult to do using mouse mesenteric vessels because of very small tissue amounts. Future studies will be designed using aortas and endothelial cell cultures to determine the mechanism of action for the effect of alcohol on vascular NO during pregnancy. Although very little is known of the relationship between alcohol and the vascular adaptations of pregnancy, it is known that alcohol influences NO production in other model systems. Exposure of bovine pulmonary arterial endothelial cells to alcohol increased NOS activity in response to endothelium-dependent vasodilators (10), and similar effects were found in isolated aortas of rats exposed chronically to alcohol (20). Greenberg et al. (16) proposed that alcohol exposure decreased inducible NOS mRNA and protein expression and increased endothelial NOS (eNOS) activity in vivo in rat and human endothelium, and Venkov et al. (41) demonstrated increased eNOS protein and mRNA levels and increased NO production in bovine aorta endothelial cells and human umbilical vein cells after alcohol treatment. However, recently it has also been shown that alcohol perfusion of normal term placental villus tissue increases eNOS protein expression but decreases NO release (22). Therefore, even though a number of studies suggest increased NOS activity with alcohol treatment, there may also be increased scavenging of NO as was suggested by Kay et al. (22). Future studies specific to the maternal system are warranted.

Determining the effects of alcohol on vascular function during pregnancy is extremely important, given that abnormalities in cardiovascular adaptations of pregnancy are associated with compromised maternal and fetal health (27, 32). Ultimately, placental blood flow and transfer may be adversely affected and this may contribute to abnormal fetal growth and development.

In the present study, pups born to alcohol-consuming dams were significantly smaller than those born to control dams. Furthermore, their brain weight/body weight ratio was lower, a common characteristic of prenatal alcohol exposure (15, 28). It should be noted that peak blood alcohol levels of the mice were indicative of moderate alcohol consumption, and low levels at the time of experimentation suggested that the observed effects were not due to high circulating plasma alcohol levels. Perhaps if dams had been killed at the time of their peak blood alcohol level, effects may have been even more pronounced.

In summary, we used a mouse model to investigate relationships among alcohol consumption, NO, and vascular adaptations of pregnancy. The data support the hypothesis that NO mediates vascular adaptations of pregnancy in mice and that alcohol consumption impairs NO-mediated vessel function. Importantly, the relationship between alcohol and vascular function in pregnancy may be more pronounced due to a greater NO modulation of vascular function compared with nonpregnant females or males. These data have important implications in determining effects of alcohol on female health and underlying factors for the adverse effects of alcohol on fetal growth and development.

**Perspectives**

It has been recognized for over two decades that alcohol abuse during pregnancy is associated with abnormal fetal growth and development, yet mechanisms underlying the detrimental effects of alcohol remain unknown. The data from this study suggest that ethanol consumption by female mice suppresses NO-mediated vasodilation in mesenteric arteries. Moreover, this ethanol effect was more pronounced in the pregnant mice.

The ability of ethanol to influence cardiovascular function during pregnancy has important implications for maternal and fetal health. For example, ethanol may reduce placental perfusion, thereby contributing to some of the negative effects of alcohol on fetal growth and development. Understanding these influences of alcohol on the vascular system could lead to new therapeutic approaches to antagonize some of ethanol’s effects during pregnancy. Furthermore, these data provide a novel approach to understanding the pathogenesis of fetal alcohol syndrome.

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