

Effects of ethanol on intraovarian nitric oxide production in the prepubertal rat

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Abstract

Nitric oxide (NO) has been shown to contribute to ovarian development and function. In non-ovarian tissues NO can be altered by ethanol (ETOH), a drug considered to be a gonadal toxin in men as well as male and female rats. The present study was undertaken to determine if some of the detrimental effects of chronic ETOH exposure on prepubertal ovarian function could be due to ETOH-induced alterations in the intraovarian NO system. Rats were implanted with intragastric cannulae on day 24 and began receiving control or ETOH diets on day 29. All rats were killed on day 34, determined to be in the late juvenile stage of development, and their ovaries and blood were collected. We analyzed the expression of the two constitutive forms of nitric oxide synthase (NOS), i.e. neuronal (n) NOS and endothelial (e) NOS, as well as the inducible (i) form of NOS protein in the ovaries of control and ETOH-treated rats by Western immunoblotting. Results demonstrate that eNOS protein increased markedly ($P < 0.02$; 140 kDa) in ETOH-treated rats compared

with controls. ETOH treatment did not alter the protein expression of nNOS (155 kDa) and only slightly increased that of iNOS (130 kDa). We also assessed NOS activity as determined by nitrite accumulation and by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline. In this regard, the ETOH-treated animals showed an increase in ovarian nitrite generation ($P < 0.05$), as well as an increase in ovarian citrulline formation ($P < 0.0001$), when compared with control animals. Along with the above described ETOH-induced increases in ovarian eNOS and NO activity, the serum levels of estradiol were concomitantly suppressed ($P < 0.001$) in the ETOH-treated rats. These results demonstrate for the first time the ETOH-induced changes in the prepubertal ovarian NO/NOS system, and suggest that these alterations contribute to the detrimental actions of the drug on prepubertal ovarian development and function.

Journal of Endocrinology (1999) **161**, 69–75

Introduction

Nitric oxide (NO) is known to be involved as a signaling molecule in a diverse array of cellular functions (Moncada *et al.* 1991, Nathan 1992). NO is produced during the enzymatic conversion of L-arginine to L-citrulline by a family of three distinct isoforms of NO synthase (NOS), which are the products of individual genes (Moncada *et al.* 1991, Nathan 1992, Wang & Marsden 1995). The two constitutive NOSs are endothelial NOS (eNOS) and neuronal NOS (nNOS), and both are calcium/calmodulin dependent for activation. The inducible NOS (iNOS) is calcium independent (Lowenstein & Snyder 1992, Nathan 1992). With regard to reproductive function, NO has been shown to be involved in the hypothalamic control of luteinizing hormone (LH) release (Bonavera *et al.* 1993, Moretto *et al.* 1993, McCann & Rettori 1996). Furthermore, various studies have suggested that ovarian NO may play a role in the ovulatory process (Ellman *et al.* 1993, Ben-Shlomo *et al.* 1994, Shukovski & Tsafrii 1994) and

that elevated NO after ovulation is inhibitory on ovarian steroidogenesis (Van Voorhis *et al.* 1994, Olson *et al.* 1996). The expression of NOS isoforms at the mRNA level has been shown to be altered during ovarian follicular development in the rat (Van Voorhis *et al.* 1995). Recently, we have shown developmental changes in the expression of ovarian NOS mRNA and protein during peripubertal maturation (Srivastava *et al.* 1997), thus further supporting the above pre- and post-ovulatory roles for the ovarian NO/NOS system.

Ethanol (ETOH), in addition to causing detrimental effects on hypothalamic-pituitary function, is also considered to be a direct gonadal toxin in men (Van Thiel *et al.* 1974, Mendelson *et al.* 1977) as well as male and female rats (for review see Dees *et al.* (1995)); thus, the possibility exists that this drug could alter or modulate specific intraovarian actions of NO. This drug has been shown to stimulate NO levels in the liver (Wang *et al.* 1995), block the response of LH-releasing hormone (LHRH) neuronal terminals to facilitatory actions of NO

(Canteros *et al.* 1995), and suppress testicular testosterone synthesis by an NO-dependent mechanism (Adams *et al.* 1993). Because of the involvement of NO in the control of ovarian development and function, and because NO synthesis has been shown to be altered by ETOH in various tissues (Davda *et al.* 1993, Wang *et al.* 1995, Naassila *et al.* 1996), the present study was conducted to determine whether the intraovarian NO/NOS system is a target for the detrimental actions of ETOH on prepubertal ovarian function.

Materials and Methods

Animals and surgery

Immature female rats used in this study were of the Sprague–Dawley line and were obtained from Charles Rivers (Boston, MA, USA). The animals were housed individually under controlled conditions of light (lights on 0600–1800 h) and temperature (23 °C). Food (Purina Laboratory Chow (lab chow)) and tap water were freely available. Each animal was anesthetized at 24 days of age with 2.5% tribromoethanol and surgically implanted with a permanent intragastric cannula by a procedure that we have described previously (Dees *et al.* 1984). Animals recovered from surgery for 5 days before the start of the experiments.

Experimental procedures

A total of 94 prepubertal female rats were used for this study. When the rats were 28 days old they were weighed and divided into three groups. The experiment began on day 29, using a diet administration regimen described previously (Dees & Skelley 1990). Because of the number of rats needed in each group, and the resulting number of surgeries, as well as the rigorous injection regimen, the following protocol was repeated twice, with each consisting of animals from all three diet groups. Briefly, group 1 consisted of rats that received a 5% ETOH liquid diet and group 2 consisted of rats that received the isocaloric control liquid diet (Bio-Serve, Inc., Frenchtown, NJ, USA). Group 3 served as an additional set of controls that consisted of rats that were cannulated and maintained on freely available lab chow and water throughout the experiment. Initially, the liquid diets for groups 1 and 2 were administered in such a manner that 7.5 ml of the respective diet were injected via the intragastric cannula (five injections of 1.5 ml each) equally dispersed over the lights-on period, then no more than 30 ml of diet was freely available (bottle) during the lights-off period. To continue providing adequate food supply for these growing animals, beginning on day 30 and again on day 32, the amount of diet administered was increased by 0.5 ml/injection. Also, the amount of diet made available each

night was increased to 35 and 40 ml respectively. These diets were administered at this latter level throughout the duration of the study. If, on occasion, an animal did not consume the full lights-off portion of the diet, the difference was made up via the gastric cannula the following day. This procedure enabled the liquid diet control animals to grow at exactly the same rate as the lab chow control animals. In addition, this method not only ensured that all liquid diet-fed animals received the same amount of diet and, thus, the same number of calories per each 24 h period, but also that all of the ETOH-treated animals received the same amount of ETOH dispersed over a 24 h period.

All animals were weighed and killed by decapitation between 1000 and 1100 h at 34 days of age. Each rat used in this study was confirmed to be in the late juvenile stage of development (just before entering the peripubertal period), based on well-defined criteria that classify the different phases of the pubertal process (Advis *et al.* 1979). These animals all had closed vaginae, small uteri (up to 94 mg) and no intraluminal fluid. Since none of the ETOH-treated rats had entered the peripubertal period, it was important that only late juvenile controls be used for comparisons with the ETOH-treated animals; hence, the few control animals that had entered the peripubertal period by day 34 were not utilized. The trunk blood collected was centrifuged at 3000 *g* for 20 min, and the serum stored at –20 °C until assayed for estradiol (E₂). The ovaries were cleaned of extraneous tissue, frozen on dry ice and stored at –70 °C until processed further for analyses. Subsequently, the ovaries were allocated into three major groups for assessments of NOS proteins, nitrite and citrulline.

Western blot analysis

Frozen ovaries were homogenized in 300 µl ice-cold homogenization buffer containing 10 mM Tris–HCl, pH 7.5; 1% SDS; 1 mM sodium vanadate; 10 µg/ml aprotinin; 10 µg/ml leupeptin; and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged at 12 000 *g* for 15 min at 4 °C and protein concentration in the supernatant was determined by the Bradford method (Bio-Rad, Richmond, CA, USA) with BSA as the standard. The preparations of total protein for the Western blot analysis of nNOS, eNOS and iNOS from ovaries were all consistent. Equal amounts of protein (100 µg) were separated on 10% SDS-PAGE under reducing conditions and electrophoretically blotted onto polyvinylidene-difluoride membranes. Additionally, a prestained molecular mass marker (Bio-Rad) was used with each run to verify the complete transfer of the protein onto the membrane. The efficiency of the protein transfer was checked as described in Sambrook *et al.* (1989) by staining the gel and membrane with Coomassie Brilliant Blue R-250 followed by destaining. In this regard, we did not

visualize any Coomassie-detectable materials on the gel. The conditions used for each respective NOS antibody incubation were consistent between replicate experiments. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline-Tween (TBT, 10 mM Tris, pH 7.5; 150 mM NaCl; and 0.1% Tween-20) overnight at 4 °C and subsequently incubated at room temperature for 90 min with the mouse monoclonal antibodies specific for either iNOS or eNOS (diluted 1:2000 in blocking buffer), or a rabbit polyclonal antibody specific for nNOS (diluted 1:500 in blocking buffer) (Transduction Labs, Lexington, KY, USA). Following incubation, membranes were washed in TBT and incubated with either anti-mouse horseradish peroxidase (HRP)-conjugated antibody for iNOS and eNOS or anti-rabbit HRP-conjugated antibody for nNOS at a dilution of 1:1000 (Amersham, Arlington Heights, IL, USA). Subsequent detection of the specific proteins was done by the enhanced chemiluminescence method (ECL Western blotting Kit, Amersham) from the different blots which were exposed to the membrane for the same period of time and signals were quantified using scanning densitometry.

Determination of NO production

For nitrite measurements, frozen ovaries were homogenized at 4 °C in five volumes of PBS (pH 7.4) and centrifuged at 10 000 *g* for 20 min. The supernatants were recentrifuged at 100 000 *g* for 15 min, followed by ultrafiltration using a 10 kDa molecular mass cut-off filter. The production of nitrite (NO₂⁻) and nitrate (NO₃⁻), stable metabolites of NO, was determined in the ultrafiltrates using Cayman's Nitrate/Nitrite assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Nitrate was enzymatically reduced to nitrite by incubation of aliquots for 3 h at room temperature in the presence of nitrate reductase and enzyme co-factors. The absorbance of nitrite was then measured colorimetrically at 550 nm with the Griess reagent using a microtiter plate reader (7500 Cambridge Tech, Watertown, MA, USA). Amounts of total nitrite were estimated by extrapolation from a standard curve of NaNO₂ obtained by enzymatic conversion of NaNO₃.

Assay of NOS activity

NOS activity was assessed by measuring the conversion of L-[U-¹⁴C]arginine to L-[U-¹⁴C]citrulline according to the method of Bredt & Snyder (1990) with some modifications. Briefly, the frozen ovaries were homogenized in 400 µl ice-cold buffer containing 50 mM Hepes, pH 7.4; 1 mM EDTA; 1 mM dithiothreitol (DTT); 1 mM PMSF; 10 µg/ml aprotinin; 10 µg/ml leupeptin; and 1 µM pepstatin A. The homogenates were centrifuged at 12 000 *g* for 15 min at 4 °C, and 300 µl supernatant were added to 200 µl reaction buffer, containing final concentrations of 50 mM Hepes, pH 7.4; L-[U-¹⁴C]arginine (250 000 c.p.m.,

specific activity 330 mCi/mmol, NEN, Boston, MA, USA); 1 mM NADPH; 1.25 mM CaCl₂; 1 mM EDTA; 1 mM DTT; and 10 µM tetrahydrobiopterin. The reaction mixture was incubated for 15 min at 37 °C and then the reaction terminated by adding 1.5 ml stop buffer (20 mM Hepes, pH 5.5; 2 mM EDTA). Samples were applied to 1 ml columns of Dowex AG50W-X8 (Na²⁺ form) that were pre-equilibrated with stop buffer. L-[U-¹⁴C]citrulline was eluted with 2.5 ml water and quantified by a Beckman (Fullerton, CA, USA) scintillation counter (Model LS1701).

Blood alcohol concentration (BAC)

BACs were analyzed by an enzymatic method (Sigma Chemical, St Louis, MO, USA) shown to be both sensitive and reliable (Redetzki & Dees 1976). The mean ± s.e.m. concentration for all ETOH-treated animals (*n*=48) was 193.3 ± 10.4 mg/dl. These BACs are not highly pharmacological, producing in the rat what is considered to be mild intoxication and, at least during this short-term study, did not appear to cause behavioral alterations.

RIA

Serum E₂ levels were measured using a kit purchased from Diagnostic Products Corp. (Los Angeles, CA, USA). The sensitivity of this assay was 2 pg/ml and the inter- and intraassay variations were 9.3 and 3.6% respectively.

Statistical analysis

Data from the various parameters studied were initially subjected to the Bartlett test for assessing homogeneity of variances. Results indicated that the differences among the standard deviations were not significant, and therefore data were then analyzed using ANOVA, with post-hoc testing using the Student-Newman-Keuls multiple range test. Because no significant differences were detected in any of the hormones or other parameters measured in the two control groups, their data were combined for presentation in the tables and figures to simplify comparative descriptions. The differences between the combined control groups and the ETOH-treated group were then analyzed using Student's *t*-test. Both methods of analysis yielded comparable results. Statistical tests were conducted using INSTAT and Prism software for the IBMPC (GraphPad, Inc., San Diego, CA, USA). *P* values < 0.05 were considered to be significantly different.

Results

As in our previous studies (Dees & Skelley 1990, Dees *et al.* 1990, Srivastava *et al.* 1995) we found no differences

Table 1 Mean \pm S.E.M. body weight, ovary weight and serum E₂ levels of the combined control and ETOH-treated animals. Note that for each measurement the ETOH-treated animals showed lower values

	Control (n=46)	ETOH (n=48)
Body weight (g)	90.9 \pm 1.57	70.1 \pm 1.90**
Ovary weight (g)	0.031 \pm 0.002	0.020 \pm 0.001**
E ₂ serum levels (ng/ml)	19.8 \pm 1.98	8.37 \pm 1.10**

***P*<0.001.

when using this diet regimen between lab chow-fed and liquid diet-fed animals with regard to growth and final body weights, as well as ovarian weights and serum E₂ levels. Specifically, the lab chow-fed control rats (*n*=23) weighed 91.1 \pm 1.9 g whereas the liquid diet-fed control rats (*n*=23) weighed 90.7 \pm 2.4 g. Ovarian weights for these control groups were also very close at 0.032 \pm 0.002 g and 0.030 \pm 0.003 g respectively. Serum E₂ levels for the lab chow-fed control animals were 21.4 \pm 2.4 ng/ml with levels of 18.3 \pm 2.0 mg/ml for the liquid diet-fed control animals. Because there were no differences between the two control groups they were combined. Table 1 shows the total number of control and ETOH-treated animals along with their respective body weights, ovarian weights and serum E₂ levels. As shown, each of the three parameters assessed was lower (*P*<0.001) in the ETOH-treated group. Additionally, the ovaries from the ETOH-treated animals were not only smaller than those of the controls, but showed much less follicular development.

Ovaries from each of the three groups were used to determine the effects of ETOH on the NO/NOS system. Western immunoblotting was used to assess the expression of the three isoforms of NOS protein. The representative autoradiogram from immunoblotting shown in Fig. 1A illustrates an immunoreactive band of 140 kDa, characteristic of eNOS detected in both control and ETOH-treated rats. Figure 1B is a composite diagram from the combined control (*n*=9) animals and the ETOH-treated animals (*n*=9) showing that ETOH administration caused an increase (*P*<0.02) in the densitometrically quantified bands of ovarian eNOS protein. Figure 2 reveals data from quantitative analysis of immunoreactive bands for nNOS and iNOS at 155 and 130 kDa respectively. Specifically, the densitometric quantitation of ovaries from control (*n*=6) and ETOH-treated (*n*=6) animals showed no differences in the expression of nNOS. Similarly, there were no differences in iNOS expression between control (*n*=11) and ETOH-treated (*n*=13) animals.

Because NOS catalyzes the formation of NO with the stoichiometric production of citrulline, we have measured both the total nitrite accumulation in control (*n*=10) and ETOH-treated (*n*=11) ovaries and the production of L-[¹⁴C]citrulline from L-[¹⁴C]arginine in control (*n*=11)

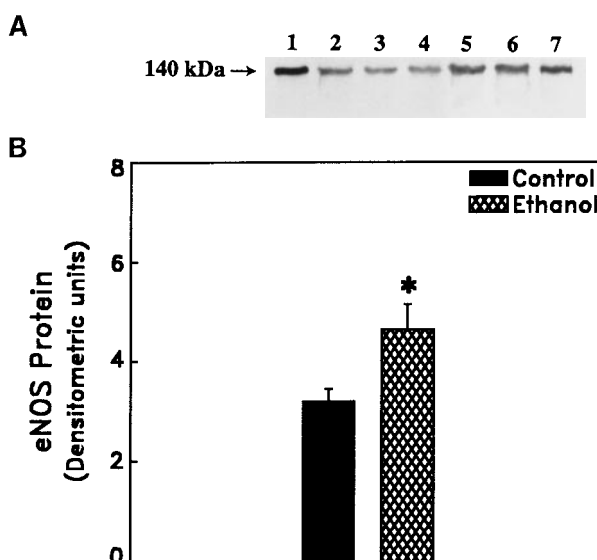


Figure 1 Assessment of the effect of chronic ETOH exposure on intraovarian eNOS protein. (A) Representative Western immunoblot of eNOS in the ovary of control (lanes 2–4) and ETOH-treated (lanes 5–7) prepubertal female rats. In lane 1 human endothelial lysate (140 kDa) was used as a positive control. Total proteins (100 μ g) were separated on 10% SDS-PAGE and electroblotted onto a polyvinylidene-difluoride membrane. The immunoblotting was performed with a mouse monoclonal anti-eNOS antibody. The band representing eNOS is seen at 140 kDa. (B) Composite graph showing the densitometric quantitation of the bands from three blots corresponding to eNOS protein. Note the protein expression increased (*P*<0.02) in the ETOH-treated rats. The respective bars depict the total of nine lanes for control and nine lanes for ETOH. The control bar represents the combined densitometric values from ovaries obtained from lab chow-fed (3.1 \pm 0.3, *n*=4) and liquid diet-fed (3.2 \pm 0.3, *n*=5) control animals. Each lane represents the pair of ovaries from each individual rat. The bars represent the mean \pm S.E.M.

and ETOH-treated (*n*=9) ovaries as indicators of NO formation. These results (Table 2) demonstrate that ETOH administration resulted in increased ovarian nitrite accumulation (*P*<0.05), as well as increased ovarian citrulline formation (*P*<0.0001) relative to their respective combined controls.

Discussion

Because of the involvement of NO in ovarian function, we hypothesized that ETOH may alter prepubertal ovarian physiology, at least in part, by exerting detrimental effects on the intraovarian NO system. Our results demonstrate the presence of the protein for all three NOS isoforms in the prepubertal ovary, and interestingly, show that ETOH did not alter nNOS protein, caused a slight increase in iNOS protein, but markedly increased the levels of eNOS protein. The fact that the expression of ovarian eNOS

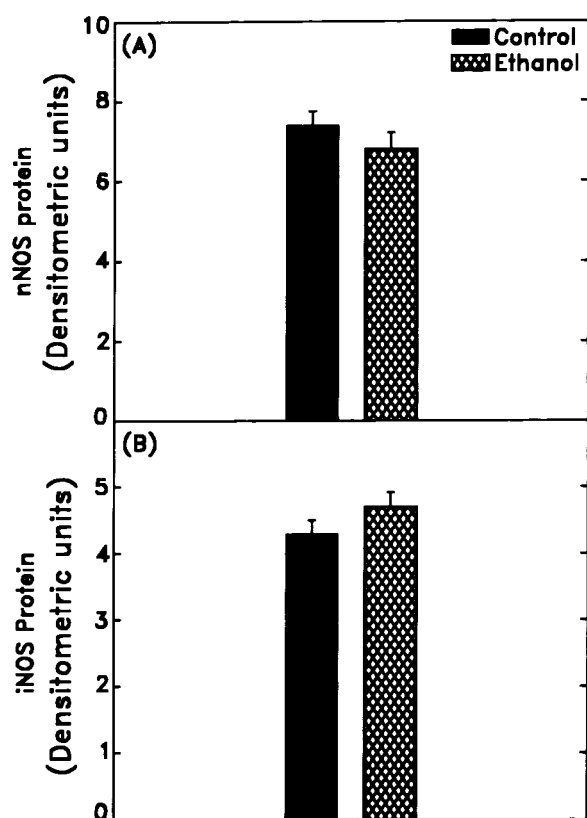


Figure 2 Assessment of the effect of chronic ETOH exposure on intraovarian nNOS and iNOS protein. Composite graphs showing the densitometric quantitations of the Western immunoblot chemiluminescence signals corresponding to nNOS protein (A) and iNOS protein (B). Note that both nNOS and iNOS protein expression did not change significantly in ETOH-treated rats compared with their respective controls. In (A), the bars depict a total of six lanes from control and six lanes from ETOH-treated rats. The control bar represents the combined densitometric values from ovaries obtained from lab chow-fed (7.2 ± 0.5 , $n=3$) and liquid diet-fed (7.5 ± 0.4 , $n=3$) animals. In (B) the bars depict a total of 11 lanes from control and 13 lanes from ETOH-treated rats. The control bar represents the combined densitometric values from lab chow-fed (4.5 ± 0.3 , $n=5$) and liquid diet-fed (4.2 ± 0.3 , $n=6$) control animals. Each lane represents the pair of ovaries from each individual rat. The bars represent the mean \pm S.E.M.

protein was affected to a greater degree than that of iNOS protein is not surprising since it has been shown by immunolocalization that a large percentage of cells express eNOS protein and only a few cells express iNOS protein in a rat ovarian cell culture system (Olson *et al.* 1996). Nevertheless, the increases which we have shown in both of these isoforms, but especially eNOS, were associated with and likely contributed to the elevated NO activity (as shown by two different methods) along with concomitantly suppressed circulating levels of E_2 . Other studies using non-ovarian tissues have indicated that chronic ETOH exposure can up-regulate NOS activity (Davda

et al. 1993, Wang *et al.* 1995). Although the mechanism(s) responsible for the increased ovarian NO after chronic ethanol exposure observed in the present study is not known, the magnitude of NO formation from NOS can be modulated by a number of factors including the level and activity of the enzyme (Xia *et al.* 1996). Whatever the mechanism, the present results are the first to show that ETOH can act on the intraovarian NO/NOS system, thus supporting the notion that this is one mechanism by which ETOH can cause detrimental actions at the level of the ovary during pubertal maturation.

Based on the present results we feel that the described ovarian effects of ETOH are due, at least in part, to a drug-induced premature elevation in intraovarian NO activity. This increase in NO during late juvenile development is associated with suppressed circulating levels of E_2 ; therefore, since it is known that ETOH-treated female rats have delayed puberty (for review see Dees *et al.* (1995)), it is conceivable that this timely elevation in NO contributes to blocking the normal rise in E_2 needed for final maturation of the central LHRH/LH releasing system. Hence, we suggest that although NO may be needed for first ovulation, a premature elevation of ovarian NO, such as we have shown here in prepubertal rats prior to first proestrus, interferes with steroidogenesis at a critical time of ovarian maturation.

It is well known that ETOH causes depressed testicular and ovarian steroidogenesis both *in vitro* and *in vivo* (Ellingboe & Varanelli 1979, Johnston *et al.* 1981, Cicero & Bell 1982, Dees *et al.* 1989, 1995). Therefore, the possibility that ETOH can induce NO production, which may then limit steroidogenesis, is important. An inhibitory action of NO on steroidogenesis is supported by several lines of evidence. Importantly, it has been shown by Adams *et al.* (1993), using NOS inhibitors, that NO mediates at least part of the ETOH-induced suppression of testicular steroidogenesis. This group suggested that ETOH may increase NO production and that the increased levels of NO are responsible for suppressed testosterone biosynthesis, possibly by NO binding to complexed iron in heme groups of steroidogenic enzymes. It has been demonstrated *in vitro* that NO decreases the action of heme-containing steroidogenic enzymes, and hence limits ovarian steroidogenesis (Olson *et al.* 1996). The inhibitory effects of NO on steroidogenesis have also been shown in cultured Leydig cells (Welch *et al.* 1995) and cultured rat granulosa cells (Dave *et al.* 1997). Further support has been shown using human granulosa-luteal cell cultures, which demonstrated that NOS inhibitors and NO donors elicited dose-dependent increases and decreases respectively in E_2 secretion (Van Voorhis *et al.* 1994). Finally, we have recently shown that ovarian NOS expression is developmentally regulated during the peripubertal period, and that the substantially elevated levels of the protein observed during first estrus and diestrus were accompanied by significantly suppressed serum levels of E_2 (Srivastava

Table 2 Mean \pm S.E.M. total ovarian nitrite and citrulline. Note that both indicators of NO production are increased. The nitrite control data represent the combined values from ovaries obtained from lab chow-fed (0.79 ± 0.12 , $n=4$) and liquid diet-fed (0.89 ± 0.09 , $n=6$) control animals. Also, the citrulline control data represent the combined values from ovaries obtained from lab chow-fed (429.5 ± 23 , $n=6$) and liquid diet-fed (443 ± 28 , $n=5$) control animals

	Control	ETOH
Total nitrite ($\mu\text{M}/\text{mg}$ tissue)	0.85 ± 0.07 ($n=10$)	$1.3 \pm 0.17^*$ ($n=11$)
Citrulline (c.p.m./mg tissue/15 min)	435.8 ± 17.2 ($n=11$)	$631.6 \pm 23.6^{***}$ ($n=9$)

** $P < 0.05$, *** $P < 0.001$.

et al. 1997). Collectively, these results demonstrate that NO is an inhibitory regulator of steroidogenesis. Also, in addition to the well-known effect of ETOH to suppress LH, we have now shown that the ETOH-induced elevations in prepubertal ovarian NO is associated with suppressed serum levels of E_2 .

In conclusion, we have shown that ETOH induces early elevations in the NO signaling system in the prepubertal ovary. Furthermore, the results suggest that this intraovarian action of the drug contributes to the decreased levels of E_2 and delayed peripubertal development associated with chronic ETOH exposure.

Acknowledgements

This work was supported by NIH Grants AA-07216 and AA-00104 with other support from the Center for Environmental and Rural Health, ES-09106.

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Received 24 July 1998

Revised manuscript received 8 October 1998

Accepted 10 November 1998