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Alcohol and Maternal Uterine Vascular Adaptations during Pregnancy – Part I: Effects of Chronic In Vitro Binge-like Alcohol on Uterine Endothelial Nitric Oxide System and Function

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Abstract

Background—Pregnancy-induced utero-placental growth, angiogenic remodeling, and enhanced vasodilation are all partly regulated by estradiol-17β-mediated activation of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) production. However, very little is known about the effects of alcohol on these maternal utero-placental vascular adaptations during pregnancy and its potential role in the pathogenesis of Fetal Alcohol Spectrum Disorders (FASD). In this study, we hypothesized that *in vitro* chronic binge-like alcohol will decrease uterine arterial endothelial eNOS expression and alter its multi-site phosphorylation activity state via disruption of AKT signaling. To study the direct effects of alcohol on uterine vascular adaptations, we further investigated the effects of alcohol on estradiol-17β-induced uterine angiogenesis *in vitro*.

Methods—Uterine artery endothelial cells were isolated from pregnant ewes (gestational day 120-130; term = 147), Fluorescence Activated Cell sorted, validated, and maintained in culture to passage 4. To mimic maternal binge drinking patterns, cells were cultured in the absence or presence of a lower (LD) or higher dose (HD) of alcohol in a compensating sealed humidified chamber system equilibrated with aqueous alcohol for 3 h on 3 consecutive days. Immunoblotting was performed to assess expression of NO system-associated proteins and eNOS multi-site phosphorylation. Following this treatment paradigm, control and binge alcohol treated cells were passaged, grown for two days, and then treated with increasing concentrations of estradiol-17β (0.1, 1, 10, 100 nM) in the absence or presence of LD or HD alcohol to evaluate estradiol-17β-induced angiogenesis index using BrdU Proliferation Assay.

Results—LD and HD binge-like alcohol decreased uterine arterial eNOS expression (P=0.009). eNOS multi-site phosphorylation activation state was altered: P^{635}eNOS was decreased (P=0.017), P^{1177} eNOS was not altered, and P^{495} eNOS exhibited an inverse U shaped dose-dependent relationship with alcohol. LD and HD alcohol decreased the major eNOS-associated protein cav-1 (P<0.001). However, the commonly implicated AKT pathway did not correlate with eNOS post-translational modifications. Assessment of uterine vascular adaptation via angiogenesis

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demonstrated that alcohol abrogated the dose-dependent proliferative effects of estradiol-17β and thus blunted angiogenesis.

Conclusions—Thus, the maternal uterine vasculature during pregnancy may be vulnerable to chronic binge-like alcohol. Altered eNOS multi-site phosphorylation also suggests that alcohol produces specific effects at the level of post-translational modifications critical for pregnancy-induced uterine vascular adaptations. Finally, the alcohol and estradiol-17β data suggest a negative impact of alcohol on estrogen actions on the uterine vasculature.

Keywords

Estrogen; Angiogenesis; FASD; eNOS; Phosphorylation; Caveolin

INTRODUCTION

Maternal alcohol consumption during pregnancy may lead to pre- and postnatal growth restriction that persists through adolescence (Day et al., 1989; Day et al., 2002). The overall whole body structural deficits are compounded by a wide range of neuroanatomical (Archibald et al., 2001), behavioral (Mattson and Riley, 1998), and memory deficits (Mattson et al., 1996) and is termed as Fetal Alcohol Spectrum Disorders (FASD). These observations in children are supported by specific phenotypes in several animal model systems especially relating to the deleterious effects of alcohol on the developing brain (Abdollah and Brien, 1995; Bonthius et al., 1992; Cudd, 2005; Weinberg et al., 2008). Candidate mechanisms include disrupted fetal neuronal cellular energetics, dysregulated developmental timing, impaired cell-cell interactions, and altered gene/protein expression (Goodlett et al., 2005). However, very little is known about disruption of maternal-fetal interface as a potential source of FASD and in particular about the effects of alcohol on maternal uterine vascular adaptations during pregnancy.

By the third trimester of gestation, uterine vascular resistance decreases by 70 fold (Rosenfeld, 1977), accompanied by extensive angiogenesis (Reynolds and Redmer, 1995; Zygmunt et al., 2003) and extracellular matrix remodeling (Osol and Mandala, 2009) resulting in a 53 fold increase in uterine blood flow compared to the nonpregnant state (Magness, 1998; Rosenfeld, 1977). These adaptations are directly controlled by concurrent elevations in estradiol-17β-induced nitric oxide (NO) production (Losordo and Isner, 2001; Magness, 1998; Magness et al., 2005; Matsubara et al., 2008; Rosenfeld et al., 1996; Rubanyi et al., 2002). We have recently demonstrated that estradiol-17β produces bi-phasic dose-dependent proliferation of uterine artery endothelial cells derived from pregnant but not nonpregnant ewes (Jobe et al., 2010). Estrogen acts on the membrane estradiol-17β receptors located on caveolar lipid rafts to induce complex regulation of endothelial NO synthase (eNOS) leading to NO production (Chen et al., 2004; Losordo and Isner, 2001). eNOS activation involves a cascade of events that involve PI3-Kinase AKT activation, eNOS posttranslational modification via multi-site phosphorylation, and eNOS translocation to non-caveolar sub-cellular compartment (Michell et al., 2002; Shaul, 2002). However, the effects of alcohol on the uterine vascular endothelial NO system during pregnancy are not well studied.

In this study, we hypothesized that chronic binge-like alcohol will decrease uterine arterial endothelial eNOS expression and alter its multi-site phosphorylation activation state via the AKT signaling pathway. To study the direct effects of alcohol on uterine vascular adaptations, we further investigated the interactive effects of alcohol and estradiol-17β on uterine angiogenesis *in vitro*. We specifically utilized ovine uterine artery endothelial cells obtained during the third trimester-equivalent of human pregnancy, a period during which alcohol-associated maladaptations have been previously reported in both ovine maternal and fetal vascular compartments (Cook et al., 2001; Falconer, 1990; Parnell et al., 2007).

MATERIALS AND METHODS

Alcohol Binging

The Animal Care and Use Committee of the University of Wisconsin-Madison approved procedures for obtaining uterine arteries from pregnant ewes (Day 120-130; term = 147) for endothelial cells isolation using collagenase digestion procedures (Bird et al., 2000). For each of the studies described, three replicates from four different pregnant ewes were utilized. Additionally, for endothelial proliferation studies, assays were performed in quadruplicates from each individual preparation. In brief, cells were purified using fluorescence activated cell sorting (FACS), devoid of vascular smooth muscle cell contamination and maintained in culture to passage 4. To mimic maternal binge drinking patterns, uterine artery endothelial cells were cultured to 70% confluence in the absence (0 mg/dl; Control, Ctrl) or presence of two doses of alcohol. To achieve a magnitude that is similar to the peak blood alcohol concentrations (BACs) obtained in previously published Fetal Alcohol Spectrum Disorders (FASD) studies performed using the sheep model system (Cudd et al., 2001; Ramadoss et al., 2008; West et al., 2001), we utilized a lower dose (LD, 300 mg/dl) group. In addition, to mimic clinically relevant abusive patterns of drinking in women of child-bearing age and those who are admitted to emergency wards (Church and Gerkin, 1988; Hammond et al., 1973; Urso et al., 1981; Wells and Barnhill, 1996), we utilized a higher dose (HD, 600 mg/dl) group. Cells were exposed to alcohol in sealed, humidified chambers equilibrated with aqueous alcohol for 3 h on 3 consecutive days (Eysseric et al., 1997; Ramadoss et al., 2010; Ramadoss et al., 2007a; Ramadoss et al., 2007b), a pattern common among drinking women of child bearing age (Caetano et al., 2006; Gladstone et al., 1996; Maier and West, 2001). Alcohol concentrations were validated using an enzymatic assay kit (Quantichrom® ethanol assay kit, BioAssay Systems, Hayward, CA; data not shown). As part of preliminary studies, cell viability was validated. At the end of the experiment, the endothelial cells were scraped and collected in a lysis buffer containing $Na_4P_2O_7$ (4 mM), HEPES (50 mM), NaCl (100 mM), EDTA (10 mM), NaF (10 mM), Na_3VO_4 (2 mM), pH (10.5), with freshly added PMSF (2 mM), Triton X100 (1% V/V), aprotinin (5 μg/ml), leupeptin (5 μg/ml), and microcystin (4 μl in 10 ml). The lysate was sonicated and centrifuged at 13,200 RPM for five minutes at 4 degree Celsius. The supernatant was assayed for protein concentration using a BCA assay kit (BCA® Protein Assay, Thermo Scientific, Rockford, IL).

Immunoblotting

Proteins (15 μg) along side of Rainbow molecular weight markers (Bio-Rad Laboratories, Inc.) were resolved on 4-20% gradient denaturing 18-well polyacrylamide gels with 0.1% SDS at 100 V for 1.5 h at room temperature before transfer onto Immobilon-P membranes at 100 V for 45 min. Non-specific binding was blocked with 5% fat-free milk in TBST (50 mm Tris-HCl, pH 7.5, 0.15 m NaCl, 0.05% Tween-20) for 120 minutes and incubated with primary antibodies in TBST + 1% BSA for 120 minutes or overnight at 4 degrees Celsius. The primary antibodies were monoclonal eNOS (1:750; BD Transduction Labs; #610312), polyclonal actin (1:3000; #4970), total caveolin-1 (1:10,000; #3251S), total AKT (1:1000; #9272), P^{1177}eNOS (1:1000; #9570S), and P^{473} AKT (1:1000; #9271S) from Cell Signaling Inc., and polyclononal P^{635}eNOS (1:1000; #07562), and P^{495}eNOS (1:1000; #07384) from Millipore Inc. After washing, the membrane was incubated with secondary antibodies (goat anti-rabbit/HRP conjugate or Sheep anti-mouse/HRP conjugate) at 1:3000 dilution and detected with the Pierce ECL or ECL plus detection kits (Thermo Scientific, Waltham, MA). Protein expression was quantified by scanning densitometry (Bio-Rad 670 scanning densitometer) and normalized to β actin.

Assessment of estradiol-17β-induced uterine vascular adaptation

Following the end of binge paradigm, cells were passaged, plated, and grown in phenol red free media (DVal EBM with 20% FBS, 100 mg/ml penicillin, and 100 mg/ml streptomycin) on a 96 well plate for 24 hours as previously described (Jobe et al., 2010). Subsequently, cells were serum starved for 24 hours and treated with 0, 0.1 nM, 1 nM, 10 nM, or 100 nM of estradiol-17β for 24 hours in the absence or presence of LD or HD alcohol. BrdU label was added at the beginning of the steroid treatment and this *in vitro* index of proliferation was utilized as an estimate of angiogenesis, a specific uterine vascular adaptation (Jobe et al., 2010; Wulff et al., 2002). Plates were read using Synergy HT Multi-Mode Microplate Reader. Proliferation results were expressed as fold increases over untreated control after subtracting the value of the blank (wells incubated without BrdU loading). Validation of cell number increase and cytoxicity after treatment with estradiol-17β was previously performed using ViaLight Plus High Sensitivity Cell Proliferation and Cytotoxicity Kit (Lonza Inc., Rockland, ME) according to manufacturer's instructions (Jobe et al., 2010). In addition, cell viability at different doses of estradiol-17β was validated as follows: after 24 hour starvation and subsequent treatment with estradiol-17β in white opaque 96 well plates (24-hours), cells were lysed with Lysis Reagent (10 mins) to extract ATP from cells. Then the appropriate amount of ATP Monitoring Reagent Plus was added (2 mins) in each well to generate luminescent signal. Plates were read using Synergy HT Multi-Mode Microplate Reader to determine luminescence and results expressed in Relative Light Units as fold increases over untreated control after subtracting the value of the blank against an ATP standard curve (Jobe et al., 2010).

Statistical Analyses

Data are expressed as mean ± SEM. Protein expression data were expressed as a ratio to actin. A two or a one way ANOVA was performed as appropriate. Further multiple pairwise

comparisons were performed when appropriate using Fisher's protected least significant difference (PLSD). The α level was established *a priori* at $P < 0.05$ for all analyses.

RESULTS

Chronic binge-like alcohol significantly decreased (one way ANOVA, $P = 0.009$) the expression of total eNOS, the rate liming enzyme for NO production in the uterine artery endothelium (Figure 1A). Compared to the Control (Ctrl) group, total eNOS expression was significantly decreased in both the Lower Dose (LD; $P = 0.009$) and Higher Dose (HD; $P = 0.005$) groups. Subsequently, eNOS multi-site phosphorylation state was examined by specifically assessing the expression of excitatory Ser 635, excitatory Ser 1177, and inhibitory Thr 495 residues (Kukreja and Xi, 2007; Mount et al., 2007). P^{635}eNOS, an excitatory phosphorylation site, and the best estimate of the activity of the enzyme in the uterine endothelium was significantly decreased by alcohol (one way ANOVA, $P = 0.017$); compared to the Ctrl group, P^{635}eNOS was significantly decreased in the LD ($P = 0.019$) and was barely detectable in the HD ($P = 0.008$) group (Figure 1B). The decrease in P^{635}eNOS was in excess of total eNOS demonstrating that eNOS was barely phosphorylated at the Ser 635 residue. Surprisingly, the excitatory P^{1177}eNOS was not different among treatment groups (Figure 1C). The classic inhibitory P^{495}eNOS exhibited an inverse U shaped relationship and was barely detectable in the Ctrl and HD groups whereas it was detectable in the LD group (Figure 1D).

The caveolar scaffolding protein caveolin-1 (total cav-1) that is bound to eNOS in its inactive state (Shaul, 2002) was significantly different (one way ANOVA, $P < 0.001$) among treatment groups (Figure 2A). Compared to the Ctrl group, total cav-1 expression was significantly decreased in the LD ($P = 0.002$) and was barely detectable in the HD ($P < 0.001$) group. Total AKT, a protein widely implicated in modulating the phosphorylation and activity states of eNOS exhibited an inverse U shaped relationship and trended to be increased in response to LD alcohol (one way ANOVA, $P = 0.063$; Figure 2B). pAKT was not significantly different among treatment groups (Figure 2C). Therefore, under the present conditions, there was no direct correlation between total AKT and eNOS expression ($R^2 = 0.122$) or their phosphorylation.

To study the direct effects of alcohol on uterine vascular adaptations, we then investigated the interactive effects of alcohol and estradiol-17β on uterine angiogenesis *in vitro*. Figure 3A depicts estradiol-17β-induced proliferative response in the LD group compared to the Ctrl. Bi-phasic dose-dependent proliferative effects of estradiol-17β that we recently reported (Jobe et al., 2010) were abolished by alcohol. The presence of LD alcohol significantly decreased estradiol-17β-induced proliferation; however, this was independent of whether the cells were previously exposed to binge-like LD alcohol. Specifically, the estradiol-17β-induced proliferation was significantly decreased in the binge-like LD alcohol group even if alcohol was not present with estradiol-17β in the system. However, this decrease was significantly lower compared to when alcohol was present with estradiol-17β. Figure 3B depicts estradiol-17β-induced proliferative response in the HD group compared to the Ctrl. Similar to the LD group, the presence of HD alcohol with estradiol-17β significantly decreased proliferation independent of whether the cells were previously

exposed to binge-like HD alcohol. However, unlike the LD group, there was no difference in the binge-like HD alcohol groups even if alcohol was not present with estradiol-17β in the system compared to when alcohol was present.

DISCUSSION

This is the first study to systematically investigate the effects of alcohol on the uterine NO system and associated vascular adaptations at the level of endothelium. The current study demonstrates that the maternal uterine vasculature is vulnerable to chronic binge-like alcohol and that these effects are in part mediated by alterations in the NO system. Alcohol decreased uterine arterial eNOS expression and negatively impacted its multi-site phosphorylation state and associated proteins cav-1 and AKT, indicating reduced eNOS enzyme activity. However, the commonly implicated AKT pathway did not directly correlate with eNOS post-translational modifications. Furthermore, assessment of uterine vascular adaptation via angiogenesis using this *in vitro* model demonstrated strong negative effects of alcohol on estradiol-17β-induced proliferation alcohol abolished dose-dependent proliferative effects of estradiol-17β and blunted angiogenesis.

Uterine vascular adaptations during pregnancy including vasodilation, angiogenesis, and extracellular matrix remodeling are in part controlled by NO (Osol and Mandala, 2009; Zygmunt et al., 2003). eNOS protein expression is dramatically increased in pregnancy compared to the nonpregnant state in the uterine artery endothelium whereas such dramatic pregnancy-associated adaptations are not observed in systemic arteries (Magness et al., 1997; Magness et al., 2001). The increase in uterine endothelial eNOS expression is accompanied by a simultaneous elevation in eNOS activity *in vitro* (Magness et al., 1997; Yi et al., 2005) and the circulating levels of NOx and cGMP *ex vivo* (Magness et al., 2001; Rosenfeld et al., 1996; Vonnahme et al., 2005). It has been previously reported that the deficiency in NO production renders the developing fetal neuronal cells more vulnerable to the toxic effects of alcohol and that the nitric oxide-cGMP-PKG pathway has a protective effect against alcohol-induced injury (Bonthius et al., 2008; Bonthius et al., 2003; Bonthius et al., 2009). However, no previous study has reported the effects of alcohol on the NO system in the maternal uterine compartment. Herein, we report for the first time that *in vitro* chronic binge-like alcohol blunts total eNOS protein expression. These data directly point to possible uterine vascular maladaptations during the third trimester-equivalent of human gestation when uterine blood flow is the highest and when the fetal brain growth velocity peaks.

The decrease in total eNOS expression was accompanied by a dramatic simultaneous alteration in the multi-site phosphorylation state markers of eNOS activation. In this study, we demonstrate that alcohol blunts phosphorylation of Ser 635, the best estimate of the activity of eNOS in the uterine vasculature. Ser 635, a well-known excitatory site, is located in the flavin mononucleotide binding domain of the enzyme (Michell et al., 2002; Mount et al., 2007) and is important for sustained NO production without requiring rises in intracellular calcium (Boo et al., 2003). The nearly complete depletion of Ser 635 phosphorylation in the HD group suggests the deleterious effects of alcohol on eNOS activity. In contrast, alcohol did not have an effect on Ser 1177 phosphorylation. Ser1177 is

a widely examined AKT-dependent site located in the reductase domain in the vicinity of the C-terminal end of eNOS, preventing the electron transfer between the two monomers (Mount et al., 2007). Although previous reports have also been skeptical of the possible role of Ser 1177 in uterine vasculature as opposed to the systemic vasculature (Cale and Bird, 2006), it could also mean that alcohol may only affect specific phosphorylation sites. We also observed that inhibitory Thr 495 phosphorylation exhibited an inverse U shaped relationship and was barely detectable in the Ctrl and HD groups whereas it was detectable in the LD group. Thr 495 is located in the calcium/calmodulin binding domain of eNOS and its phosphorylation inhibits eNOS-calmodulin interaction and the activity of the enzyme (Cale and Bird, 2006; Mount et al., 2007). In summary, alcohol adversely affects eNOS multi-site phosphorylation suggesting lowered enzyme activity state and uterine NO production.

In resting conditions, eNOS is primarily located in specialized protein-rich plasmalemmal invaginations called caveolae, where its activity is inhibited by direct binding to the scaffolding protein cav-1 (Michell et al., 2002; Sessa, 2004; Shaul, 2002). The scaffolding domain (amino acids 61–101) and to a lesser extent the C-terminal tail (amino acids 135–178) of cav-1 directly interact with eNOS (Garcia-Cardena et al., 1997). In this study, we observed that total cav-1 was significantly decreased in the LD alcohol group and was barely detected in the HD group. Our data support earlier studies where alcohol has been demonstrated to disrupt the caveolae by caveolar cholesterol/lipid depletion and disruption of caveolar assembly of proteins (Mao et al., 2009; Ramadoss et al., 2010; Ronis et al., 2007). Collectively, these data suggest that chronic binge-like alcohol has deleterious effects on the caveolar lipid rafts. Thus, we hypothesize that alcohol will negatively impact the temporal and spatial partitioning of eNOS between caveolar and non-caveolar sub-cellular compartments. We observed that total AKT expression trended to be biphasic, with higher levels in the LD group compared to the Control and the HD group. However, the phosphorylation of Ser 473 residue of AKT was not different among groups. The protein kinase AKT is the most cited kinase protein in the context of eNOS phosphorylation and is an important downstream target of PI3 kinase (Dimmeler et al., 1999; Michell et al., 1999; Michell et al., 2002; Mount et al., 2007; Tanimoto et al., 2002; Tsang et al., 2004). In the systemic endothelium, inhibition of the AKT pathway or mutation of the AKT site on eNOS protein (at Ser 1177) attenuates the serine phosphorylation and prevents the activation of eNOS (Dimmeler et al., 1999). In contrast, it has been reported previously that AKT may not play a significant role in uterine vascular eNOS activity (Bird et al., 2003). Therefore, the next step will be to investigate if alcohol mediates altered eNOS multi-site phosphorylation via alternate signaling cascades like the ERK and the P38 MAPK pathway.

Finally, we further investigated uterine vascular adaptation by assessing estradiol-17β-mediated endothelial proliferation. We specifically studied mitogenic responses to estradiol-17β, a hormone that is significantly elevated in pregnancy (Carnegie and Robertson, 1978; Magness, 1998) and acts via specifically upregulating uterine eNOS expression and activity (Vagnoni et al., 1998). Further, we recently demonstrated that estradiol-17β increased uterine artery endothelial cell proliferation in a bi-phasic dose-dependent pattern (Jobe et al., 2010). In the present study, we observed that alcohol abolished the mitogenic effects of estradiol-17β. However, in both HD and LD groups, the

presence of alcohol with estradiol-17β significantly decreased proliferation independent of whether the cells were previously exposed to binge-like alcohol. Unlike the effects of HD alcohol, despite prior binge-like LD alcohol exposure, estradiol-17β induced proliferation was less affected when LD alcohol was not present together with estradiol-17β in the system. This observation is easily attributable to dose-dependent effects of alcohol or the partial recovery of proliferative response in the LD compared to the HD group. Collectively these data support the notion that chronic binge-like alcohol inhibits uterine angiogeneic indices. In support of the fact that all models of Intrauterine Growth Restriction (IUGR) developed to date are associated with some degree of reduction in uterine blood flow (Reynolds et al., 2006), our data suggests that FASD-associated IUGR must also be accompanied by a severely compromised uterine vascular function. Whether this is mediated by abrogation of the estradiol-17β mediated uterine angiogenesis or reductions in vasodilator NO production remains to be determined. Other studies do show an interaction between estradiol-17β and alcohol as well as a sexually dimorphic differential response. Associations between alcohol and estradiol-17β have also been noted with reference to the cytochrome P450 CYP19 aromatase activity; perinatal alcohol exposure increases aromatase activity in select brain regions in male but not female rats (McGivern et al., 1988). In humans, elevated estradiol-17β is associated with increases in acetaldehyde levels in women but not men (Eriksson et al., 1996). In summary, we demonstrate here that there is a negative effect of alcohol on estradiol-17β-induced NO associated vascular endothelial proliferation in the uterine endothelium.

We conclude that chronic binge alcohol modulates specific uterine vascular adaptations during pregnancy including the NO system. Effects of alcohol range from altered uterine endothelial expression of total eNOS to disrupted eNOS multi-site phosphorylation and altered expression of major NO-associated proteins including cav-1 and AKT. Furthermore, alcohol negatively modulates estrogen-induced uterine angiogenesis. As part of this publication series, we will investigate the mechanistic perspectives underlying alcohol-induced alterations in uterine vascular adaptations in the pregnant state in an attempt to understand the role played by the maternal uterine vasculature in the pathogenesis of FASD.

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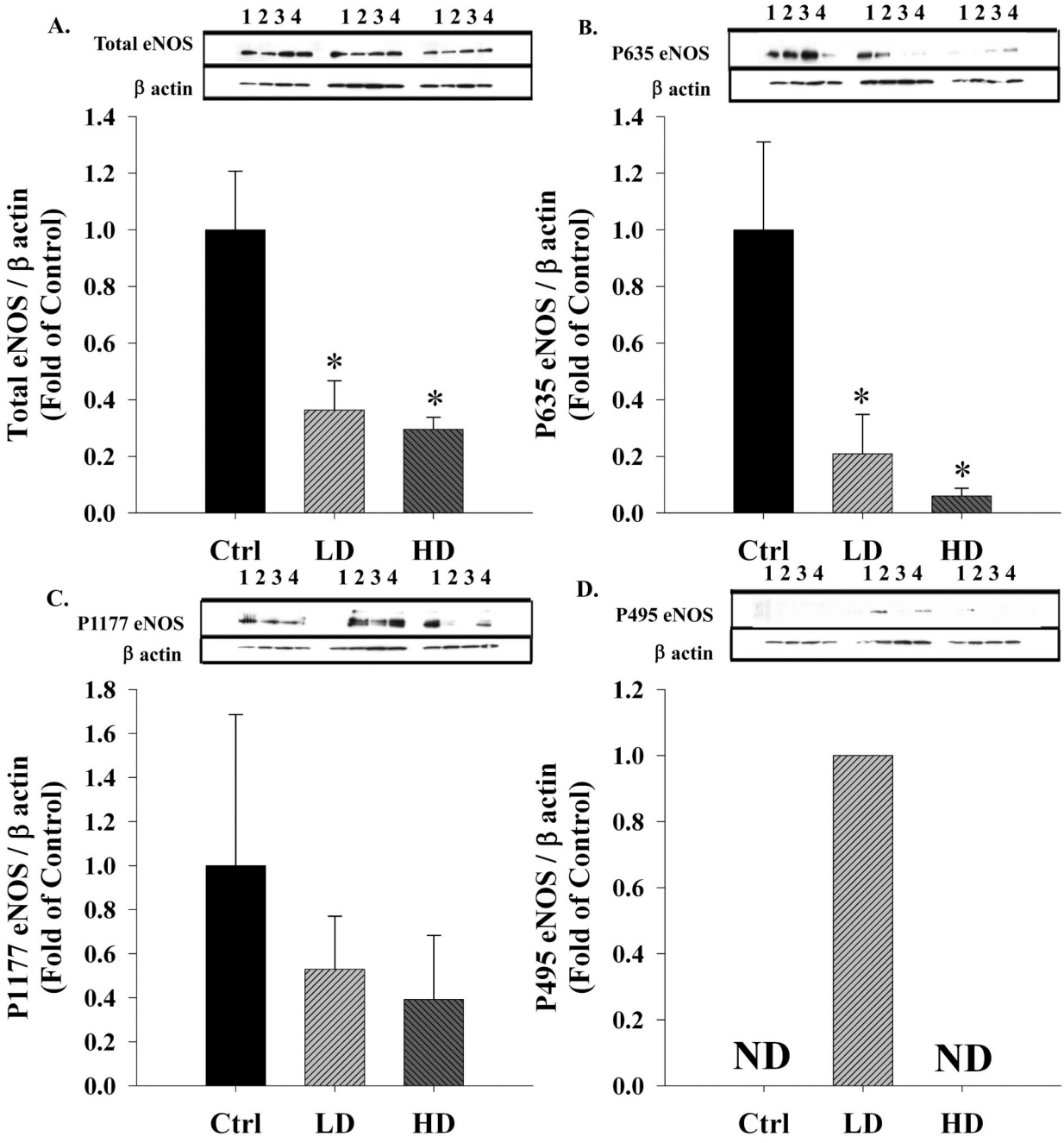

Figure 1. Effect of chronic binge-like alcohol on eNOS expression and multi-site phosphorylation

(A) Compared to the control (Ctrl) group, total eNOS expression was significantly (*, $P < 0.05$) decreased in both the lower dose (LD) and higher dose (HD) groups. (B) The excitatory P^{635}eNOS was significantly decreased in the LD group and was barely detectable in the HD group. (C) The excitatory P^{1177}eNOS was not different among treatment groups. (D) The classic inhibitory P^{495}eNOS exhibited an inverse U shaped relationship and was barely detectable (ND) in the Ctrl and HD groups whereas it was detectable in the LD group. Optical Density (OD) data are expressed as a ratio to β actin OD.

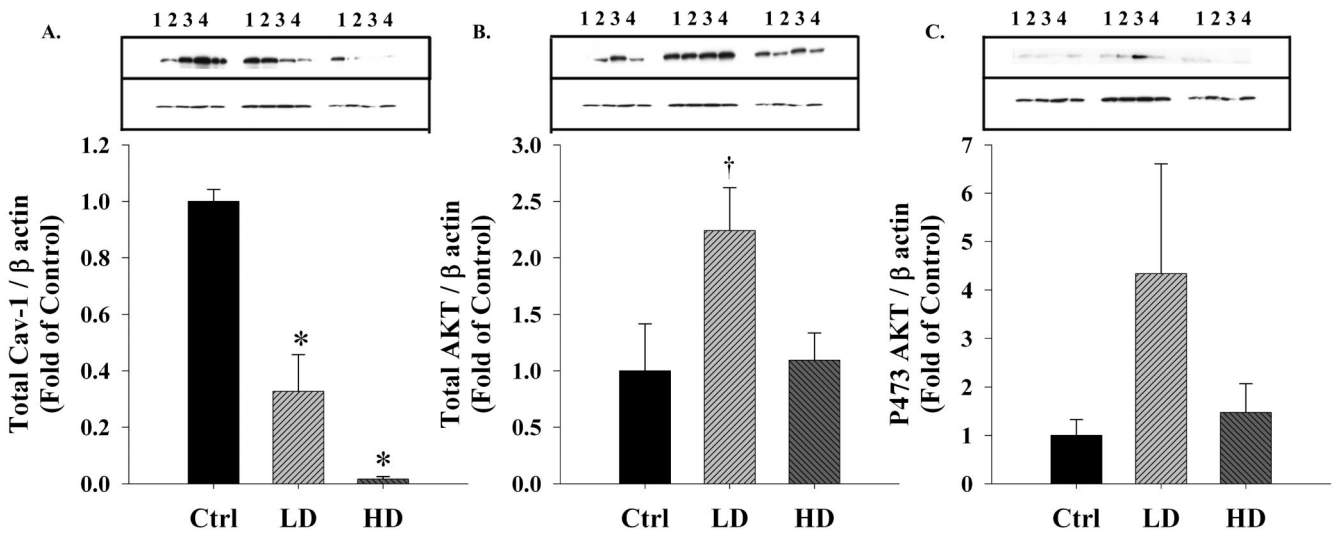

Figure 2. Effect of chronic binge-like alcohol on eNOS associated proteins

(A) Compared to the control (Ctrl) group, the caveolar scaffolding protein cav-1 was significantly (*, $P < 0.05$) decreased in both the lower dose (LD) and higher dose (HD) groups. (B) Total AKT, a protein widely implicated in the eNOS phosphorylation exhibited an inverse U shaped relationship and trended (†) to be elevated in response to LD alcohol ($P = 0.063$). (C) However, activated pAKT was not different among treatment groups. Optical Density (OD) data are expressed as a ratio to β actin OD.

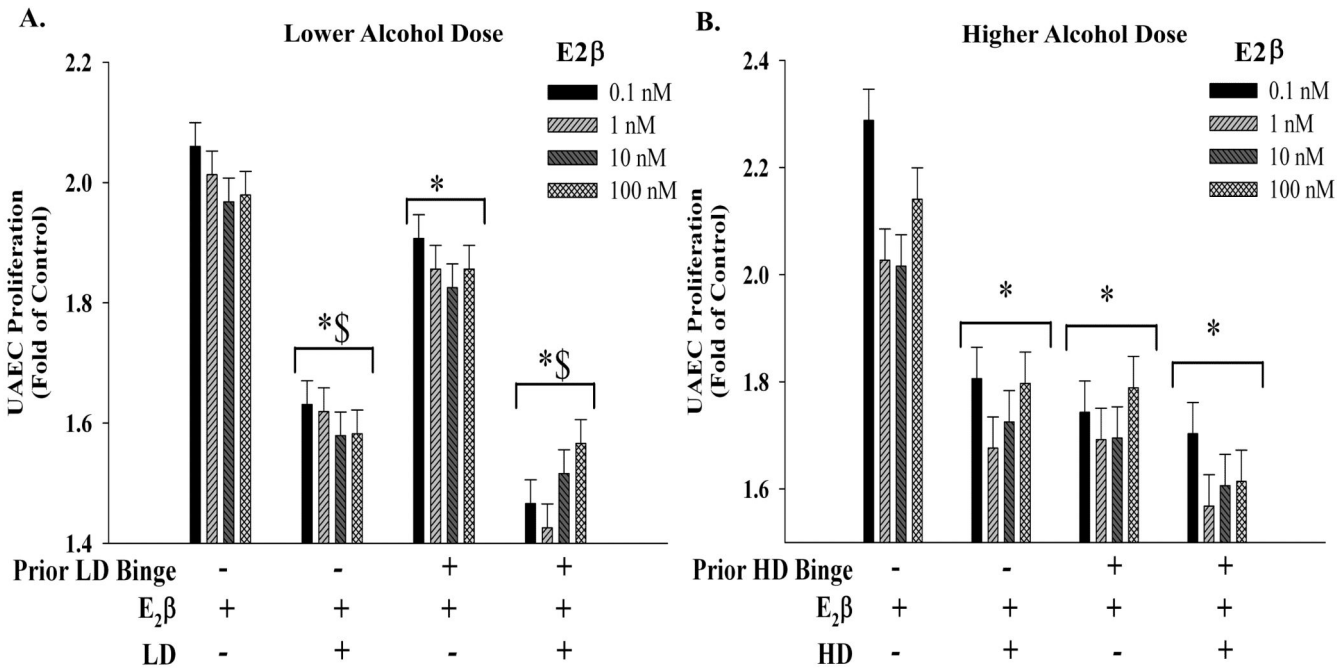

Figure 3. Interactive effects of estradiol-17β and alcohol on uterine arterial endothelial proliferative function

(A) Estradiol 17β-induced uterine artery endothelial cell (UAEC) proliferative response in the lower dose (LD) alcohol group compared to the controls (Ctrl). Dose-dependent proliferative effects of estradiol-17β that we previously reported (Jobe et al., 2010) were abolished by alcohol. The presence of LD alcohol with estradiol-17β significantly (*) decreased proliferation independent of whether the cells were previously exposed to binge-like LD alcohol. Specifically, the estradiol-17β-induced proliferation was significantly decreased in the binge-like LD alcohol group even if alcohol was not present with estradiol-17β in the system. However, this decrease ($) was significantly lower compared to when alcohol was present with estradiol-17β. (B) Estradiol-17β-induced proliferative response in the higher dose (HD) group compared to the Ctrl. Similar to the LD group, the presence of HD alcohol with estradiol-17β significantly decreased (*) proliferation independent of whether the cells were previously exposed to binge-like HD alcohol. However, unlike the LD group, there was no difference in the binge-like HD alcohol groups even if alcohol was not present with estradiol-17β in the system compared to when alcohol was present.