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Interactive effects of In Vitro Binge-Like Alcohol and ATP on Umbilical Endothelial Nitric Oxide Synthase Post-Translational Modifications and Redox Modulation

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Abstract

Alcohol dysregulates the regulation of reproductive vascular adaptations. We herein investigated chronic binge-like alcohol effects on umbilical endothelial nitric oxide synthase (eNOS) multi-site phosphorylation and related redox switches under basal (unstimulated) and stimulated (with ATP) states. Alcohol decreased endothelial excitatory ^{Pser1177}eNOS ($P < 0.001$), whereas excitatory ^{Pser635}eNOS exhibited a main effect of alcohol ($\downarrow P = 0.016$) and ATP ($\uparrow P < 0.001$). Alcohol decreased ^{Pthr495}eNOS ($P = 0.004$) levels, whereas inhibitory ^{Pser116}eNOS exhibited an alcohol main effect in both basal and stimulated states ($\uparrow P = 0.005$). Total eNOS was reduced by alcohol ($P = 0.038$). In presence of ATP, alcohol inhibited ERK activity ($P = 0.002$), whereas AKT exhibited no alcohol effect. Alcohol main effect on S-nitroso-glutathione reductase ($\downarrow P = 0.031$) and glutathione-S-transferase ($\downarrow P = 0.027$) were noted. Increased protein glutathiolation was noted, whereas no alcohol effect on GSH, GSSG, NOX2 or SOD expression was noted. Thus, alcohol effects on multi-site post-translational modifications and redox switches related to vasodilatory eNOS underscore the necessity for investigating alcohol-induced gestational vascular dysfunction.

Keywords

Endothelium; eNOS; ATP; vascular; FASD

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Introduction

Alcohol is a well documented teratogen. Developmental alcohol exposure leads to a range of deficits in the developing fetus including growth restriction, neuroanatomic, behavioral, and cardiovascular deficits collectively termed as Fetal Alcohol Spectrum Disorders (FASD) [1, 2]. Among these deficits, the fetal vascular anomalies have been less investigated. Alcohol administration during pregnancy is reported to decrease placental blood flow in the rats [3] and decrease umbilical blood flow in the sheep [4]. Further, pups born to rats exposed to alcohol during pregnancy exhibit decreased vasorelaxation [5]. However, the mechanisms underlying these deficits are not well studied.

Nitric oxide (NO), derived from the catalytic activity of endothelial NO synthase (eNOS) is an important vasodilator [6]. Many alcohol studies on eNOS have been performed in adult non-pregnant human or animal tissues; for instance, *in vitro* acute treatment of alcohol (0.1%, 3h) increased bovine aortic endothelial NO [7], whereas alcohol at 4 g/kg for 12 weeks decreased rat aortic eNOS expression, NO levels, and impaired vasorelaxation [8]. Intravenous infusion of alcohol in male and non-pregnant female rabbits [9] as well as in healthy human volunteers [10] dose-dependently decreased levels of exhaled NO. Among studies on fetoplacental cells, *in vitro* acute alcohol exposure (100 or 200 mM, 2h) decreased placental villi NO and cyclic GMP levels [11]. In human umbilical vein endothelial cells, acute alcohol treatment exhibited dose-dependent effects on NO levels [12]. However, none of these studies have investigated effects of a binge-like pattern of alcohol administration or determined the effects of alcohol on eNOS multi-site phosphorylation.

eNOS has four main phosphorylation sites, namely, ^{Pser1179}eNOS, ^{Pser635}eNOS, ^{Pthr495}eNOS, and ^{Pser116}eNOS. Out of these sites, ^{Pser1179}eNOS and ^{Pser635}eNOS are excitatory, whereas ^{Pthr495}eNOS and ^{Pser116}eNOS are inhibitory [13-15]. An agonist (example, shear stress, ATP, estradiol 17 β , etc.) activates the enzyme eNOS via differentially modifying the eNOS multi-site phosphorylation sites [16]. Therefore, we herein for the first time have comprehensively investigated the effects of chronic binge-like alcohol on eNOS multi-site phosphorylation system as well as the related redox balance system under basal (unstimulated) and stimulated (in the presence of the endogenous eNOS activating agonist ATP) states. Our novel findings delineate alcohol-induced impairment of major post-translational modifications associated with umbilical eNOS activity regulation including multi-site phosphorylation, redox switches, and underlying signaling systems.

1. Materials & Methods

2.1 Alcohol Dosing Paradigm

As previously described, we closely mimicked a dose and paradigm of alcohol exposure reported in several animal and human studies [17-20]; in brief, a two week binge-like alcohol exposure paradigm except for two days while passaging (3h/day, 130 mM; human umbilical vein endothelial cells, Lonza) was followed using a sealed compensating system, a pattern of exposure common among women who abuse alcohol during pregnancy [19-25].

This dose mimics clinically relevant abusive patterns of drinking in women of child-bearing age and those who are admitted to emergency wards [17, 26-28]. On the last day of the *in vitro* experiment, the endothelial cells (primary, 3-4 replicates per treatment group) were serum starved for 4h, treated with or without the endogenous eNOS activating agonist ATP (100 μ M, 5 min) [16], scraped and collected in a lysis buffer (Cell Singaling Inc., 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin) containing phosphatase inhibitor cocktail (Sigma, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, leupeptin hemisulfate salt, pepstatin A), phosphatase inhibitor cocktail 2 (Sigma, Na₃VO₄, sodium molybdate, sodium tartrate, imidazole), and phosphatase inhibitor cocktail 3 (Sigma, cantharidin, (-)-p-bromolevamisole oxalate, calyculin A).

2.2 Immunoblotting

Solubilized protein was quantified using a modified Lowry assay procedure (Bio-Rad Laboratories, Inc., Hercules, CA), and equal volume or mass of protein were separated by size on 4-20% polyacrylamide gels (150 V, 42 min; Mini Protean II, Bio-Rad Laboratories, Inc.) alongside Rainbow molecular weight markers (Bio-Rad Laboratories, Inc.) before transfer to Immobilon P membranes (100 V, 1 h). The Immobilon P membranes were probed using the enhanced chemiluminescence reagent detection system, as described by Amersham Pharmacia Biotech (Arlington Heights, IL), and exposed to Hyperfilm (Amersham Pharmacia Biotech). Antibodies to ^{Pser1179}eNOS, cav-1, ^{Pser473}AKT, total ERK, total AKT, and β -actin were obtained from Cell Signaling Technologies Inc., total eNOS from BD Transduction laboratories, GP91Phox (NOX₂) and all other phosphorylated eNOS antibodies from Millipore Inc. Antisera for ^{Pthr202/tyr204}ERK (Promega Inc.), S-nitrosoglutathione reductase (GSNOR, Lifespan Inc.), glutathione-S-transferase (GST, Pierce Inc.), superoxide dismutase (Abcam Inc.), nitrotyrosine (Sigma), and glutathiolated protein (PSSG, Virogen Inc.) were also utilized for analyses.

2.3 Measurement of glutathione (GSH) and glutathione disulphide (GSSG) using HPLC

Reduced (GSH) and oxidized (GSSG) glutathione were extracted from cell lysates containing 50 μ g of protein using 1:1 solution of 12 mM iodoacetic acid and 1.5 M perchloric acid to make the final volume to 250 μ L and vortexing for 30 seconds. 125 μ L of 2M potassium carbonate was added to each tube and vortexed for 30 seconds. Supernatant from the previous step was derivatized to S-carboxymethyl glutathione using 25 mM iodoacetic acid in the presence of 2-mercaptatethanol (for the detection of total glutathione) or 40 mM sodium borate (for the detection of reduced glutathione). Using a modified procedure [29, 30], samples were run separately on HPLC system for the detection of reduced and total glutathione using a Supelco C₁₈ guard column (4.6 mm x 5 cm, 20-40 μ m; Supelco® Cat # 59644) and a Supelco C₁₈ column (4.6 mm x 15 cm, 3 μ m; Supelco® Cat # 58985). Model 717 plus WISP Autosampler was programmed to mix 25 μ L of derivatized sample (or standard) with 25 μ L of the *o*-phthalaldehyde (OPA) reagent for 1 min and then injected the derivatized solution into the HPLC column without any delay. Total run time of 16 min was set at the flow rate of 1.1 ml/min. Initial solvent conditions were 97% A, 3% B run for 1 min. A gradient to 86% A, 14% B was run from 1.1 to 6.5 min. From 6.6 to 9 min,

the conditions were maintained at 0% A, 100% B and returned to 97% A, 3% B from 9.1 to 16 min. Mobile phase A was 0.1 M sodium acetate and mobile phase B was HPLC-grade methanol (Fisher Scientific®, Cat # A452-4). 2475 Multi wavelength Fluorescence Detector (Waters® Milford, MA, USA 01757) was set at 220 nm excitation between 0 to 6 min, at 340 nm excitation between 6 to 12 min, and at 220 nm excitation between 12 to 16 min. The setting of excitation at 220 nm before 6 min and after 12 min was designed to suppress fluorescence due to other amino acids that react with OPA. Emission was set at 450 nm between 0 to 16 min and gain of the detection was set at 10. Reduced and total glutathione were quantified on the basis of authentic standards (GSH; Sigma® Cat # G 6529 and GSSG; Sigma® Cat # G 4376) using the Millennium-32 software and workstation.

2.4 Statistical analysis

Interactive effects of alcohol and ATP were analyzed using a two-way ANOVA. Further pairwise comparisons were performed when appropriate using Fisher's protected least significant difference. Level of significance was established at $P < 0.05$. $0.05 < P < 0.1$ were considered trends.

2. Results

Alcohol significantly decreased umbilical endothelial excitatory $\text{P}^{\text{Ser1177}}\text{eNOS}$ by 22.6% ($P < 0.001$), whereas an alcohol effect was not observed in the presence of the agonist ATP (Figure 1). In contrast, the excitatory $\text{P}^{\text{Ser635}}\text{eNOS}$ exhibited a significant main effect of both alcohol ($\downarrow P = 0.016$) and ATP ($\uparrow P < 0.001$). Similar to $\text{P}^{\text{Ser1177}}\text{eNOS}$, the caveolin-1 (cav-1)-binding-associated $\text{P}^{\text{Thr495}}\text{eNOS}$ exhibited a significant interaction; alcohol decreased levels of $\text{P}^{\text{Thr495}}\text{eNOS}$ by 28.1% ($P = 0.004$), whereas an alcohol effect was not observed in the presence of the agonist ATP. In contrast, the inhibitory $\text{P}^{\text{Ser116}}\text{eNOS}$ exhibited a main effect of alcohol in both basal and stimulated states ($\uparrow P = 0.005$) and the effect of ATP trended towards significance ($P = 0.068$). The two well studied signaling systems associated with eNOS phosphorylation, namely $\text{P}^{\text{Thr202/tyr204}}\text{ERK}$ and $\text{P}^{\text{Ser473}}\text{AKT}$ were studied for underlying mechanisms. ERK activity was not altered in the basal state, whereas in the presence of ATP, alcohol inhibited ERK activity by 23.8% ($P = 0.002$). A significant effect of ATP was further noted independent of alcohol exposure. Activated AKT was not altered by alcohol in the basal state, whereas in the presence of ATP, alcohol significantly increased its levels by 35.1% ($P = 0.005$) showing that alcohol does not alter eNOS multi-site phosphorylation via the classical AKT site. Total eNOS was significantly reduced by alcohol ($P = 0.038$). Furthermore, following chronic alcohol programming, its levels increased stoichiometrically and concomitantly with a trend in cav-1 scaffolding protein levels in response to ATP treatment (Figure 2). However, no changes were noted in the expression of total ERK and AKT. Furthermore, following sucrose density gradient centrifugation of ovine uterine artery endothelial cells, the caveolae enriched fractions were isolated and studied using immunoblotting; excitatory $\text{P}^{\text{Ser635}}\text{eNOS}$ was decreased in both the caveolar and non-caveolar and inhibitory $\text{P}^{\text{Thr495}}\text{eNOS}$ was decreased in the caveolar enriched fractions following chronic binge alcohol exposure (Unpublished observations, J. Ramadoss, D.B. Chen, and R. R. Magness). In summary, alcohol interacts with ATP in a complex manner to alter eNOS multi-site phosphorylation status.

We then investigated GSNOR, an enzyme responsible for denitrosylation of nitrosylated proteins including eNOS (Figure 3). A significant main effect of alcohol ($\downarrow P=0.031$) was noted independent of ATP showing that alcohol decreases denitrosylation. Further, GST was similarly decreased in the alcohol groups ($P=0.027$) independent of ATP. In contrast neither GSH nor GSSG were altered by alcohol. We further tested this by measuring NADPH oxidase 2 (NOX2) and superoxide dismutase (SOD) responsible for superoxide homeostasis and determined that NOX2 was not altered by alcohol and SOD was barely detectable in any of the treatment groups. Examination of glutathiolated proteins (PSSG) showed bands at ~140 kDa with significantly increased expression in the alcohol group by 72.2% ($P=0.005$). Similarly, tyrosine-nitratation revealed a band at ~140 kDa and its expression trended lower in the alcohol group ($P=0.084$, data not shown). In summary, these data show that alcohol produces specialized redox post-translational modifications that are related to the eNOS enzyme system.

3. Discussion

The current report is the first study on the effects of alcohol on umbilical endothelial eNOS post-translational modification and multi-site phosphorylation state. Three major conclusions can be gleaned from this study. First, *in vitro* binge-like alcohol has detrimental effects on all major eNOS multi-site phosphorylation sites in the primary umbilical endothelial cells (see summary model, Figure 4). Second, there is an interactive effect of alcohol and the endogenous eNOS activating agonist ATP on eNOS multi-site phosphorylation state in these cells. Third, alcohol modulates eNOS-related redox switch post-translational modifications in these cells. These alcohol effects on multi-site post-translational modifications of the major vasodilator system enzyme eNOS has significant implications on fetal umbilical vascular function in FASD.

In vitro binge alcohol exposure leads to umbilical vascular maladaptations

The current study demonstrates negative effects of alcohol on umbilical endothelial vasodilator system enzyme eNOS and its multi-site post-translational modification. These findings support earlier work showing that umbilical blood flow significantly decreased from 572 ± 74 ml/min to 391 ± 74 ml/min following intravenous infusion of alcohol in ewes administered with 1g alcohol/min over 1h and the decreases were maintained for 2h post-infusion [4]. Determination of regional blood flow using microspheres in an earlier rodent study that utilized progressively increasing alcohol (10% to 20% V/V before gestation and 30% V/V *in utero*) doses demonstrated that placental blood flow decreased by ~52% in the alcohol group [3]. Other alcohol studies on the maternal uterine endothelium show similar effects including alcohol-induced decreases in 17β -estradiol induced uterine endothelial cell proliferation, decreases in 20 angiogenesis-associated genes, and impairment of the physiologic conversion of the maternal uterine vasculature within the mesenteric triangle in rats [22, 31, 32]. In summary, alcohol effects on umbilical endothelial eNOS system underscores the necessity for studying alcohol-induced alterations in gestational vascular function and adaptations.

Impact of chronic alcohol on endothelial eNOS-Cav-1 complex

Chronic alcohol decreases the expression of the enzyme eNOS, that catalyzes the production of NO, a major vasodilator. Alcohol programs the endothelial cells such that treatment with eNOS activating agonists like ATP increases the inhibitory cav-1 scaffolding protein expression concomitant with proportional increases in eNOS levels. Interestingly, control endothelial cells were not programmed for altered protein expression upon ATP treatment. Over-expression of cav-1 leads to decreased NO production and cav-1 interaction with eNOS negatively regulates basal NO release [33]. Thus, eNOS and cav-1 are significant targets of chronic alcohol exposure [34-36] in the umbilical vascular endothelial cells.

Interactive effects of alcohol and ATP on eNOS multi-site phosphorylation

Alcohol decreases phosphorylation level of the well known eNOS phosphorylation site ^{Pser1177}eNOS in the basal but not ATP-stimulated state. ^{ser1177}eNOS is a widely investigated reductase domain excitatory phosphorylation site close to the C-terminus [13] and is shown to be stimulated by mutation of serine to aspartate, and inhibited by mutating the residue to alanine [37]. This site is phosphorylated by bradykinin [38], adiponectin [39], epidermal growth factor [40], histamine [40], thrombin [40], vascular endothelial growth factor [41], and shear stress [42]. ATP is an endogenous vasodilator that binds to endothelial purinergic P2Y receptors and activates eNOS via both signaling cascade(s) activation as well as via an increase in levels of intracellular $[Ca^{2+}]_i$ [16]. In this study, we found that in the umbilical endothelial cells, an alcohol effect in the presence of ATP was observed for two of the four phosphorylation sites. Ongoing studies in our lab will block different ATP-stimulated kinases as well as inhibit $[Ca^{2+}]_i$ transients to study underlying pathways by which alcohol mediates altered eNOS multi-site phosphorylation under basal and stimulated states. Alcohol also decreased ^{thr495}eNOS phosphorylation levels, a site located in the eNOS Ca^{2+} -calmodulin binding domain [43]. ^{thr495}eNOS phosphorylation is essential for eNOS-calmodulin interaction and for localizing the enzyme within the caveolar lipid rafts and making it available for extracellular agonists [13, 44]. The decreases in the caveolar ^{Pthr495}eNOS levels were confirmed by sucrose density gradient centrifugation showing that although eNOS translocates from the membrane, it is still inactive and probably also inaccessible for further agonist stimulation. Although the most reported signaling molecule for ^{ser1177}eNOS phosphorylation is AKT [38], the current report shows that alcohol disrupts its phosphorylation via other mechanisms that may include CaM-dependent kinase II (CaMKII) [45] and AMPK [39] which will be investigated in future. In summary, alcohol-induced reduction of ^{ser1177}eNOS phosphorylation might directly result in decreased eNOS enzyme activity because mutation of this residue to aspartate reduces both basal and stimulated NO production [13, 46]. However, alcohol-induced alterations to umbilical endothelial ^{Pthr495}eNOS levels may decrease NO production via the effects on subcellular localization of the enzyme within the caveolar lipid rafts [47, 48].

Alcohol further differentially altered the phosphorylation levels of the two other major sites with significant decreases in the levels of excitatory ^{Pser635}eNOS and increases in inhibitory ^{Pser116}eNOS in the absence as well as presence of ATP. ^{ser635}eNOS is an excitatory site in the CaM autoinhibitory domain [13] and its phosphorylation peaks between 5 and 10 min with sustained levels up to 30 min [44]. NO production from

phospho-mimetic (S635D)-expressing cells was significantly higher than that of both control and non-phosphorylatable (S635A) constructs in both basal and increased $[Ca^{2+}]_i$ states [49]. The sucrose density gradient centrifugation data further support decreases in excitatory P^{ser635} eNOS in both caveolar and non-caveolar subcellular fractions. Similarly, inhibitory P^{ser116} eNOS is the only site identified in the oxygenase domain of the enzyme [13]. For example, a phosphorylation-deficient mutant of eNOS in which serine 116 was mutated to alanine demonstrated enhanced enzyme activity compared with the wild-type [50]. Furthermore, we did not find that the commonly implicated ERK pathway also related to the eNOS multi-site phosphorylation levels. In summary, alcohol-induced reduction of P^{ser635} eNOS phosphorylation might directly result in decreased eNOS enzyme activity because phosphorylation of this site directly relates to sustained endothelial NO production [13, 44]. Further, alcohol-induced increases in P^{ser116} eNOS levels will also contribute to decreased NO production as mimicking dephosphorylation of this site via mutation to alanine has been reported to increase the enzyme activity [50].

Impact of chronic alcohol on redox modifications

For the first time, we herein show that alcohol impacts eNOS-related redox post-translational modifications. In the absence or presence of ATP, alcohol decreased the key denitrosylating enzyme GSNOR (also called alcohol dehydrogenase 3). GSNOR regulates both protein (PSNO) and non-protein (GSNO) S-nitrosylation homeostasis [51] and exhibits non-hyperbolic saturation chemical kinetics with ethanol [52] (Figure 4). Further, the observation that alcohol decreased GST expression independent of ATP concomitant with our previous reports that thioredoxin is decreased demonstrate important alcohol effects on protein S-glutathiolation redox post-translational modification and homeostasis of protein thiols (PSH/PSOH) [21, 53-55]. In fact, we confirmed this by probing for PSSG and detected strong bands at ~140 kDa and we reason that eNOS is glutathiolated by alcohol. S-glutathiolation of eNOS results in formation of disulphide bonds between cysteine thiol groups and GSH [56] leading to decreased NOS activity [56, 57]. Furthermore, probing for protein tyrosine nitration yielded bands near 140 kDa showing that alcohol decreased protein nitration. In contrast, alcohol did not affect GSH, GSSG, NOX2, or SOD showing absence of impairment of superoxide homeostasis. Together, we show that alcohol alters eNOS-related specialized redox switches in umbilical endothelial cells.

Perspectives

Prenatal alcohol exposure is a major public health concern as it causes a spectrum of lifelong, debilitating consequences for the affected child. The lack of a clinically relevant viable treatment option for the disorder is likely due to alcohol's complex mechanisms of action involving multiple organ systems. Although FASD studies so far have primarily focused on the neurodevelopmental sequelae, the current report illustrates major impact of alcohol exposure on the coordination and regulation of umbilical vascular eNOS adaptations. In fact, the current report supports the hypothesis that alcohol fosters a negative synergy by impacting multiple post-translational modifications, and thus impairs the critical eNOS vasodilatory system leading to vascular dysfunction. Thus, a real cure for FASD would likely involve targeting multiple systems including the fetoplacental and umbilical vascular endothelial compartment.

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Highlights

- Chronic binge alcohol exposure dysregulates the exquisite coordination and regulation of umbilical endothelial vascular adaptations.
- Binge-like alcohol has detrimental effects on all four major eNOS multi-site phosphorylation sites.
- There is an interactive effect of alcohol and the endogenous eNOS activating agonist ATP on eNOS multi-site phosphorylation state.
- Alcohol modulates eNOS-related redox switch post-translational modifications
- Major role for the reproductive vascular system in pathogenesis of Fetal Alcohol Spectrum Disorders.

Figure 1.

A. *In vitro* chronic binge alcohol exposure significantly decreased umbilical endothelial excitatory Pser1177eNOS ($P<0.001$) in the basal but not ATP-stimulated state; **B.** The excitatory Pser635eNOS exhibited a significant main effect of both alcohol ($P = 0.016$) and ATP ($P<0.001$). **C.** Alcohol decreased levels of Pthr495eNOS ($P=0.004$) in the basal state, whereas an alcohol effect was not observed in the presence of the agonist ATP; **D.** The inhibitory Pser116eNOS exhibited a main effect of alcohol in both basal and stimulated states ($P=0.005$), whereas the ATP effect trended towards significance ($P=0.068$). **E. & F.** The two well studied signaling systems Pthr202/tyr204ERK and Pser473AKT associated with eNOS phosphorylation showed absence of effect of alcohol in basal state, whereas alcohol-inhibited ERK activity ($P=0.002$) and increased AKT activity ($P=0.005$) in the presence of ATP. *, $P<0.05$; #, $0.05<P<0.1$; 3-4 replicates per treatment group.

Figure 2.

A. & B. Total eNOS was significantly decreased following *in vitro* chronic binge alcohol exposure ($P=0.038$), whereas following chronic alcohol programming, its levels increased stoichiometrically and concomitantly with a trend in cav-1 scaffolding protein in response to ATP treatment. **C. & D.** No changes were noted in the expression of total ERK and AKT. In certain cases, gels were run in replicates and were used for quantification. *, $P<0.05$. #, $0.05<P<0.1$; 3-4 replicates per treatment group.

Figure 3.

A. & B. Following *in vitro* chronic binge alcohol exposure, S-nitroso-glutathione reductase (GSNOR, $P=0.031$) and glutathione transferase (GST, $P=0.027$) exhibited a significant main effect of alcohol independent of ATP. **C. & D.** Neither reduced (GSH) nor oxidized (GSSG) glutathione were altered by alcohol. **E. & F.** NADPH Oxidase 2 (NOX2) was not different among groups. Examination of glutathiolated proteins (PSSG, $P=0.005$) showed bands at ~140 kDa and was significantly increased following alcohol exposure in the basal state. *, $P<0.05$; 3-4 replicates per treatment group.

Figure 4. Schematic illustration of chronic binge alcohol-induced dysregulation of the umbilical endothelial eNOS system. The figure depicts the biochemical targets (*, molecules measured) of alcohol. Chronic binge alcohol produces detrimental effects on all major eNOS multi-site phosphorylation sites, and eNOS-related redox switch post-translational modifications. Refer Discussion for details and abbreviations.