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High Throughput Caveolar Proteomic Signature Profile for Maternal Binge Alcohol Consumption

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Abstract

Currently, no single marker is sensitive and specific enough to be considered a reliable biomarker for prenatal alcohol exposure. To identify a proteomic signature profile for maternal alcohol consumption, we performed high throughput proteomics on maternal endothelial caveolae exposed to moderate binge-like alcohol conditions. In these specialized lipid ordered microdomains which contain a rich assembly of proteins, we demonstrate that moderate binge-like alcohol resulted in a distinctive maternal caveolar proteomic signature with important proteins being dramatically decreased/knocked out in the alcoholic profile. These proteins span from histones and basic structural proteins like tubulin α to proteins involved in trafficking, deubiquitination, cell signaling, and cell-cell adhesion. The profile also suggests an important role for the mother and the utero-placental compartment in the pathogenesis of Fetal Alcohol Spectrum Disorders (FASD). These data demonstrate that the caveolar proteomic signature created by alcohol shows a promising direction for early detection of FASD.

INTRODUCTION

Early *in utero* detection of FASD is highly desired for commencing therapeutic intervention and for stopping alcohol use for the remainder of pregnancy (Bearer, 2001). Biomarkers developed for maternal alcohol consumption include increases in: 1) blood gamma-glutamyltransferase, 2) blood carbohydrate-deficient transferrin, 3) mean corpuscular volume, 4) blood hemaglobin-acetaldehyde adduct, 5) specific fatty acid ethyl esters (FAEE) and ethyl glucuronide in meconium and hair, and 6) neonatal urine dolichols (Hannuksela et al., 2007; Bearer et al., 2004). Although these are useful indicators of heavy alcohol consumption, no single marker is sensitive and specific enough to be considered a reliable biomarker for prenatal alcohol exposure (Bearer, 2001; Stoler et al., 1998; reviewed by Bearer et al., 2004).

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The advancing field of proteomics offers promise for developing state of the art biomarkers that can detect extremely subtle physiologic changes associated specifically with alcohol use (Bearer et al., 2004). It is critically important to identify pregnant women who drink, to start them on suitable nutritional/ pharmacologic/ behavioral therapies and also test the putative efficacy of these treatments by repeated measurements of these markers over the course of pregnancy. So far, few investigators have focused attention on proteomic analyses designed to establish potential biomarkers for prenatal alcohol exposure (Bearer et al., 2004). Robinson and colleagues (1995) reported eight serum proteins whose concentrations differed significantly between the FASD and control children. However, this study neither utilized high throughput proteomics, nor did it conclusively identify a reliable marker that can predict prenatal alcohol exposure.

In this study, we specifically exploited the caveolae which are specialized lipid ordered microdomains containing assemblies of proteins (receptors, channels, signaling complexes). The caveolae are found in many cell types including the red blood cells (RBCs) and the endothelial cell (Ozuyaman et al., 2008; Parton and Simons, 2007). We hypothesize that high-throughput proteomic analysis will identify a distinctive proteomic signature profile for maternal alcohol consumption in these membrane structures. The first theory that alcohol might disrupt the caveolae comes from the observation that alcohol affects major signalosomes that are located in the caveolae. Ronis et al., 2007 speculated that this action is due to alcohol-induced caveolar cholesterol/lipid depletion. Recently, Mao et al., 2009 demonstrated that alcohol disrupts the interaction of proteins with the caveolar scaffolding protein caveolin-1 (cav-1), resulting in dissociation of these complexes from the lipid rafts. In this study, we specifically utilized fully validated ovine maternal uterine artery endothelial cells as the caveolae are best characterized in this cell type. Maternal cells were isolated during a period when blood flow to the uteroplacental unit is ~25 fold greater than the non-pregnant state (Magness, 1998). This is also a period when alcohol decreases uterine perfusion (Falconer, 1990), reduces fetal growth (Ramadoss et al., 2006) and produces fetal neuronal loss and behavioral deficits (Goodlett and Eilers, 1997; Ramadoss et al., 2008; Thomas et al., 1996). In addition to signature profile development, the strategical utilization of endothelial cells from the uterus served a twin purpose of providing some novel mechanistic insights on the role of intra-uterine environment in disorders associated with prenatal alcohol exposure. Finally, the ovine system is ideal for this purpose as the third-trimester equivalent of human gestation occurs *in utero* in this species (Cudd, 2005).

METHODS

Alcohol Binging

The Animal Care Committee 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). Cells were further 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) or presence of alcohol (150 mg/dl) in sealed, humidified chambers equilibrated with aqueous alcohol for 3 h on 3 consecutive days (Eysseric et al., 1997; Ramadoss et al., 2007a; Ramadoss et al., 2007b). Five replicates in each group were utilized. Cell viability was validated by trypan blue exclusion microscopy, Calcein AM imaging, and immunoblotting. Trypan blue stained cell count demonstrated that the number of viable cells in the control and alcohol groups were not different (control, 891,250 ± 11,433; alcohol, 834,000 ± 49,784; $p = 0.73$). We also found that uncleaved caspase 3 was unaltered and cleaved caspase 3 was not detectable. Further, 150 mg/dl is easily achieved in mothers of children with FAS (Church and Gerkin, 1988) and in women who abuse alcohol (Urso et al., 1981).

This level of alcohol produces a decrease in ovine maternal uterine blood flow by nearly 20% (Falconer, 1990), and produces fetal neuronal deficits (Parnell et al., 2007).

Caveolar Isolation

Caveolar isolation was performed as described previously (Liao et al., 2009; Song et al., 1996). Cells were collected in a 0.5 ml sodium carbonate buffer (pH 11) containing phosphatase inhibitors and protease inhibitors. A 5–35% discontinuous sucrose gradient was formed above (3.5 ml of 35% sucrose/0.5 ml of 5% sucrose; in MBS containing 250 mM sodium carbonate). The samples were centrifuged at 116,000 × g for 16–20 hours in a SW55Ti rotor (Beckman Instruments, Palo Alto, CA). Fractions (0.5 ml) were collected from the top of the tube. A light scattering band confined to the 5–35% sucrose interface was observed when enriched with caveolar membranes.

Proteomic Analysis

Caveolar proteins were extracted by precipitation with equal volume of 15% ice-cold TCA and incubated for 1hr on ice, centrifuged for 10 min at 16,000g and pellets washed (3×) with ice-cold acetone. Pelleted proteins were denatured in 10µl of 6M Urea/ 100mM NH_4HCO_3 for 10min then diluted to 50µl for tryptic digestion with: 1µl of 25mM DTT, 7µl Acetonitrile, 22µl MilliQ water and 10µl trypsin solution (20ng/µl Trypsin Gold from PROMEGA Corp. in 25mM NH_4HCO_3). Reaction was terminated by acidification with 2.5% Trifluoroacetic Acid to 0.5% final. Peptides generated from digestion were directly loaded for nanoLC-MS/MS analysis. Peptides were analyzed by nanoLC-MS/MS using the Agilent 1100 nanoflow system (Agilent, Palo Alto, CA) connected to a hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap, Thermo Fisher Scientific, San Jose, CA) equipped with a nanoelectrospray ion source. Capillary HPLC was performed using an in-house fabricated column with integrated electrospray emitter essentially as described previously (Martin et al., 2000) except for the use of 360µm × 75µm fused silica tubing. Sample loading and desalting were done at 10uL/min with the loading solvent delivered from an isocratic pump. The column was packed with 5µm C_{18} particles (Column Engineering, Ontario, CA) to approximately 12cm. Sample loading (8µl) and desalting were achieved using a trapping column in line with the autosampler (Zorbax 300SB-C18, 5µM, 5×0.3mm, Agilent). HPLC solvents were as follows: Loading: 1% (v/v) acetonitrile (ACN), 0.1M acetic acid; A: 0.1M acetic acid in water, and B: 95% (v/v) ACN, 0.1M acetic acid in water. Gradient elution was performed at 200nL/min and increasing %B in A of 0 to 40 in 200 min, 40 to 60 in 20 min, and 60 to 100 in 5 min. The LTQ-Orbitrap was set to acquire MS/MS spectra in data-dependent mode. Raw MS/MS data was searched against NCBI non-redundant Bos Taurus amino acid sequence database using in-house Sequest search engine with methionine oxidation, asparagine and glutamine deamidation and cysteine carbamidomethylation as variable modifications. Protein abundance score was determined by normalizing the spectrum counts within untreated control and alcohol groups using Scaffold (Proteomics Software Inc.). Specificity was set *a priori* at an abundance score value ≥ 20 for control state and the protein identification probability at $p < 0.05$.

Validation

Proteomic profiles were confirmed by immunoblotting for cav-1, and endothelial nitric oxide synthase (eNOS). Cav-1 was selected because it is the major caveolar scaffolding protein and is a mandatory marker for the caveolae. Signaling of the endothelial marker eNOS is compartmentalized within the caveolae under control conditions (Chen et al., 2001). In brief, all fractions (1–13) from sucrose gradient centrifugation were loaded (16 ul/lane) on 4–20% polyacrylamide gels and probed for cav-1 (Cell Signaling Technologies) and eNOS (BD Transduction Laboratories) following transfer.

RESULTS

Representative MS/MS spectra of cav-1 and eNOS illustrate a nearly complete Y ion series assignment (figure 1A). B ion series confirm peptide assignment. All major signals in the spectra are explained by the assigned sequences. Sequest cross correlation scores for cav-1 and eNOS are 4.58 and 4.08 respectively. Cav-1 and eNOS parent mass error are −0.39 PPM and −1.8 PPM respectively. Chronic-binge alcohol exposure resulted in decreases in the abundance of caveolar proteins related to cell-cell adhesion (by 29%), cell function (by 53.3%), cell signaling (by 57.2%), deubiquitination (by 51.5%), histones (by 13.2%), nitric oxide regulation (by 60.2%), structure (by 36.4%), and transport/trafficking (by 53.3%) compared with those in the control state (figure 1B). The caveolar proteomic signature profile in response to binge-like alcohol is depicted in figure 2. Caveolar profile of proteins related to cell-cell adhesion/interaction consisted of platelet endothelial cell adhesion molecule (Pecam1), integrin α5, fibronectin receptor (Fibronectin rp), integrin β1, vascular endothelial (VE) cadherin, integrin β3, synaptosomal-associated protein 23 (SAP23), serpin 1, catenin α1 and fibronectin. Alcohol altered this signature pattern with the most dramatic decrease seen with Pecam1 abundance (by ~64%). The only protein that exhibited a marked increase in response to alcohol was fibronectin (~164%). Several proteins normally associated with cell function and/or cellular organelles (e.g. golgi, endoplasmic reticulum, and mitochondria) exhibited a distinct profile in response to alcohol including hexokinase, CD109, ribophorin 2 (RPN2), dolichyl-diphosphooligosaccharide-protein glycotransferase (DDOPG), translocon-associated protein δ (TAP δ), inner mitochondrial membrane protein (mito membr), cytochrome b5 reductase 3 (CYB5R3), transmembrane emp24 transporter (emp24), prohibitin 2, ribophorin 1 (RPN 1), prohibitin, and nicotinamide nucleotide transhydrogenase (NNT). Alcohol altered this profile dramatically with hexokinase, a glycolytic enzyme decreasing by ~84% and CD 109, a protein associated with cell differentiation / transformation decreasing by ~73%. We note that it has already been demonstrated that presence of cellular organelle-associated proteins in caveolae is not a result of contamination as these proteins are integral parts of caveolae (McMahon et al., 2006). Numerous cell signaling associated proteins were found in the caveolae including Ca^{++} transporting ATP synthase (Ca++ transport), ATP synthase β unit, Ca^{++} ATPase, ATP synthase, $Na^{+}K^{+}$ATPase, RAS family protein RAB1A, G αi, calnexin (canX), and stator of ATP synthase (St ATP synthase). Alcohol dramatically altered this profile with Ca^{++} transporter decreasing by 84% and other ATP synthases or ATPases decreasing by nearly 40–70%. We also found the major deubiquitination enzyme ubiquitin specific peptidase 2 (USP2) in the caveolae which was decreased by ~52% by alcohol. It is noteworthy that three histone proteins were found in great abundance in the caveolae and alcohol reduced H2A by ~53%. The caveolar proteins related to nitric oxide signaling including heat shock protein (HSP)70 V, cav-1, and eNOS were dramatically decreased by binge-like alcohol exposure. However, prostacyclin synthase (PGI2 syn) located in the caveolae was marginally increased by ~7% in response to alcohol. A number of structural proteins including tubulin α3c, lamin B1 (LMNB1), cytoskeletal-associated protein 4 (CAP4), myoferlin, leucine rich repeat (LRR), vimentin, actin γ1, and tubulin β were identified in the caveolae and alcohol produced a dramatic alteration in this profile with α tubulin being knocked out of the caveolar profile. In contrast, tubulin β increased ~18% above control. Finally, important solute carriers, trafficking proteins and transporters were detected in the caveolae, including protein translocator SEC61, SNARE 22 (SEC22), lectin-mannose binding protein (LMAN2), voltage dependent anion channel 2 (VDAC2), adenosine translocator (Adenine tr), voltage dependent anion channel 1 (VDAC1), and phosphate solute carrier 25A3 (SLC25A3). Of these, SEC 61 was dramatically decreased (~90%) in the alcoholic profile.

Immunoblot validation of cav-1, the major caveolar scaffolding protein, and eNOS, an enzyme that is compartmentalized to the caveolae confirmed high throughput proteomic

findings (figure 3). Further, whole cell eNOS level was comparable between high throughput proteomics and Western blotting (eNOS decrease: with proteomics, by 58.54%; with immunoblotting, by 57%) (data not shown). These alcohol responses for eNOS and Cav-1 are both novel observations.

DISCUSSION

Though a wealth of information can be gleaned from the proteomic profile, we will focus on seven major and salient observations. ***First***, binge-like alcohol results in a distinctive caveolar proteomic signature profile with several abundant caveolar proteins being dramatically decreased or knocked out by alcohol. Though the mechanisms underlying the effects of alcohol on the caveolar proteome are unknown, these results are in agreement with earlier studies on the overall negative effects of alcohol on the caveolar rafts (Mao et al., 2009; Ronis et al., 2007; Wang and Abdel-Rahman, 2005; Wood et al., 2001). ***Second***, a moderate concentration of alcohol (150 mg/dl) has a substantial impact on the caveolar proteome. The concentration of alcohol utilized in this study is also clinically relevant compared to many FAS studies utilizing other model systems which assess alcohol-induced neuro-structural/ behavioral deficits at doses > 300 mg/dl (Livy et al., 2003). Further, the magnitude of caveolar lipid raft disruption may be proportional to the level of alcohol insult and since a number of proteins are dramatically decreased or knocked out by a moderate alcohol concentration, it suggests that the proteome will reflect alcohol exposure much beyond the time of insult. However, these temporal studies have not yet been performed. ***Third***, the proteins that decreased dramatically or knocked out in the alcoholic profile span from basic structural proteins like actin γ to proteins involved in trafficking, cell signaling, and cell-cell adhesion. ***Fourth***, we observed a nearly 164% increase of fibronectin, a multifunctional extracellular matrix protein that forms a kind of scaffolding in which the cells are embedded (Tuma and Casey, 1998). In adult non-pregnant humans, chronic alcohol has been demonstrated to increase levels of fibronectin in the lung (Burnham et al., 2007) and the perivenular zone of the liver (Savolainen et al., 1995). In rats, short term alcohol leads to accumulation of fibronectin in the heart (Vendemiale et al., 2001). In pregnant women, increased plasma fibronectin levels is utilized as a marker for gestational hypertensive disorders (Paarlberg et al., 1998) and fetal fibronectin is associated with the onset of labor (Cunze et al., 1996). Finally, these data may also have physiologic implications on uterine endothelial cell shape and function (Grinnell et al., 1982). ***Fifth***, the fact that alcohol dramatically decreases the caveolar abundance of histone H2A family proteins provides novel cues about epigenetic effects of alcohol. ***Sixth***, dramatic alteration in all caveolar proteins associated with nitric oxide regulation, and numerous ATP-related proteins are noteworthy with reference to regulation of uterine blood flow during pregnancy. The immunoblot not only validates the proteomic data but also clearly demonstrates movement of eNOS away from its caveolar “home” with every bout of alcohol, a finding that suggests significant utero-placental vascular effects of alcohol during the third trimester-equivalent of human gestation. Further, dramatic decrease of many ATP-associated proteins may exacerbate alcohol-induced impaired vasodilation as ATP is a powerful physiologic agonist for nitric oxide release in the uterine vascular bed during pregnancy (Bird et al., 2003). The fact that nitric oxide also regulates the intricate coordinated growth and remodeling of the entire uterine circulation, as well as the creation of a placenta suggests a very important role for the maternal utero-placental vascular compartment in the pathogenesis of FASD. Finally, the direct impact of the nitric oxide system on the fetal compartment is substantiated by reports that deficiency in nitric oxide production renders the developing fetal neuronal cells more vulnerable to the toxic effects of alcohol and that the nitric oxide-cGMP-PKG pathway has protective effect against alcohol-induced injury (Bonthius et al., 2003; 2008; 2009). ***Seventh***, alcohol-induced dramatic reduction of USP2, the major deubiquitinating enzyme may provide mechanistic

perspectives underlying caveolar protein depletion and/or degradation as USP2 depletion has been documented to significantly enhance protein degradation (Tirat et al., 2005).

These data demonstrate for the first time that the caveolar proteomic signature for alcohol consumption shows a promising direction for early detection of FASD. As proof of principle, this study exploited the unique advantages of the well characterized endothelial caveolae for biomarker(s) development. A logical extension of this study would be to demonstrate this caveolar signature pattern for maternal alcohol consumption in the RBCs, a blood-borne cell type that is readily accessible. Follow up studies will also include characterization of caveolae from uterine and umbilical endothelial cells from alcohol exposed ewes as well as from human umbilical cord endothelial cells which are easily obtained from mothers following parturition. Further, *in vivo* studies employing patterns of ethanol consumption that more closely resemble human drinking may produce very different protein signatures than that observed *in vitro* studies using relatively acute ethanol exposure paradigms.

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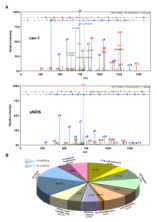

Figure 1.

(A) Representative MS/MS spectra of cav-1 and eNOS illustrate a nearly complete Y ion series assignment. The B ion series confirm peptide assignment. All major signals in the spectra are explained by the assigned sequences. Sequest cross correlation scores for cav-1 and eNOS are 4.58 and 4.08 respectively. Cav-1 and eNOS parent mass error are −0.39 PPM and −1.8 PPM respectively. (B) Chronic binge-like alcohol exposure resulted in dramatic decreases in the abundance of endothelial caveolar proteins related to cell-cell adhesion, cell function, cell signaling, deubiquitination, histones, nitric oxide/vascular tone, structure, and transport/trafficking. Pie chart slices bound by solid lines represent the caveolar protein abundance in control state. The shaded subset in each slice represents the caveolar protein abundance in response to chronic binge alcohol.

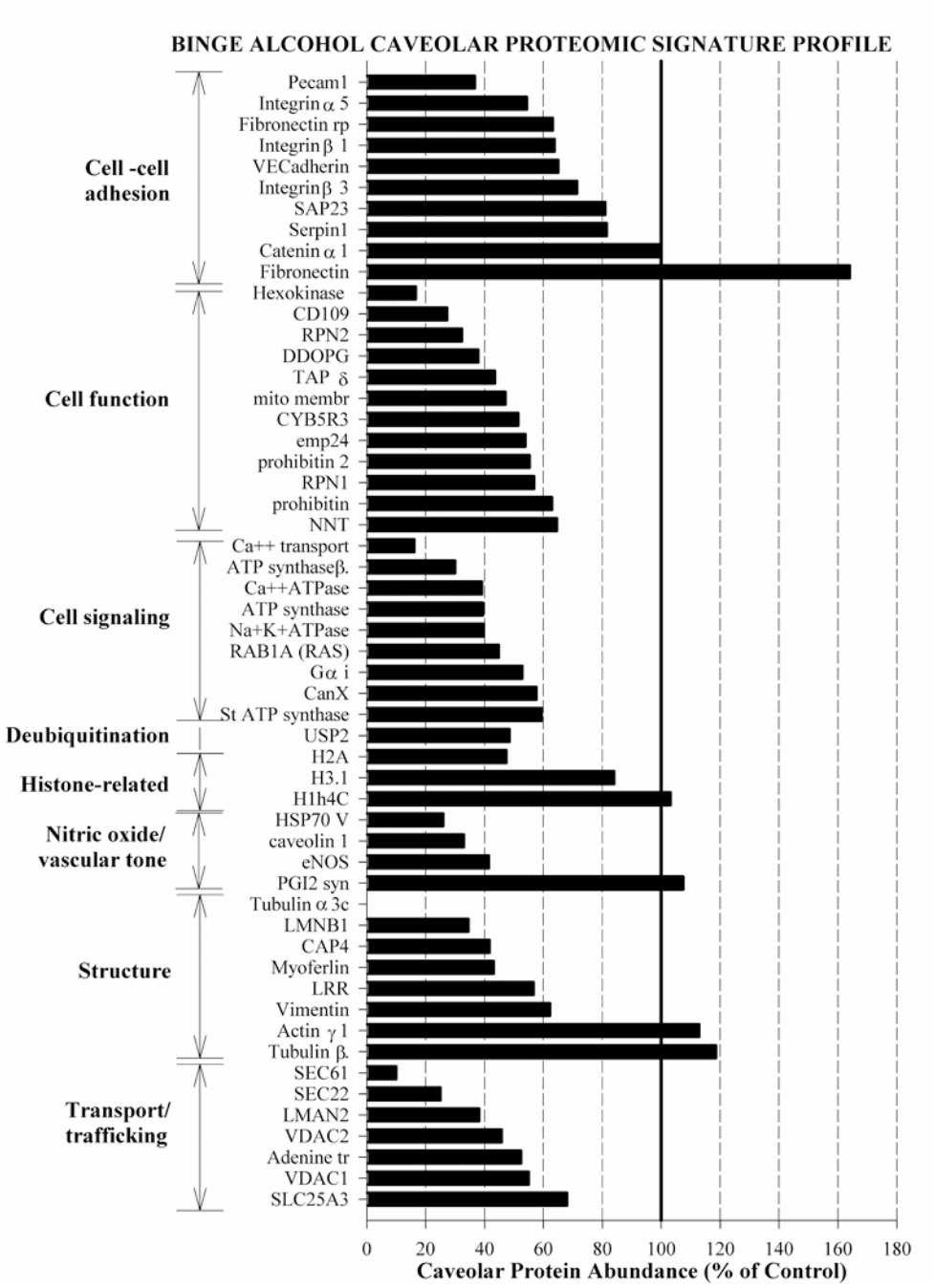

Figure 2.

Chronic binge-like alcohol exposure resulted in a distinctive endothelial caveolar proteomic signature profile that included proteins related to cell-cell adhesion, cell function, cell signaling, deubiquitination, histones, nitric oxide/vascular tone, structure, and transport/ trafficking. Profile details and abbreviations are described in the Results section. The caveolar protein profile is depicted as % of control.

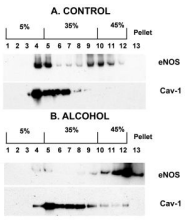

Figure 3.

Western immunoblot analysis of gradient density centrifugation of subcellular fractions fractions prepared from (A) control (0 mg/dl) and (B) binge alcohol (150 mg/dl) treated uterine arterial endothelial cells from pregnant ewes. Under control conditions, cav-1 and eNOS were both predominantly located in the caveolar pool (lanes 4–5; figure 2A) whereas endothelial nitric oxide synthase was entirely depleted from this caveolar pool in response to binge-like alcohol (figure 2B).