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2-D DIGE Uterine Endothelial Proteomic Profile for Maternal Chronic Binge-Like Alcohol Exposure

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Abstract

Little is known about alcohol effects on the utero-placental compartment during pregnancy. For the first time, we utilized 2-D DIGE quantitative proteomics to evaluate the role of the uterus in Fetal Alcohol Spectrum Disorders (FASD) pathogenesis. Uterine artery endothelial cells were isolated from pregnant ewes, FAC sorted, validated, and maintained in culture. To mimic maternal binge drinking patterns, cells were cultured in the absence or presence of alcohol (300 mg/dl) in a compensating sealed humidified chamber system equilibrated with aqueous alcohol for 3 h on 3 consecutive days for two weeks. CyDye switch combined with 2-D DIGE followed by MALDI-TOF and tandem MS/MS were utilized. Validation was performed using Western immunoblot analysis. Chronic binge-like alcohol significantly ($P < 0.05$) decreased 30 proteins and increased 19 others. Gene-enrichment and functional annotation cluster analysis revealed significant enrichment ($P < 0.05$) in three categories: glutathione S transferase, thioredoxin, and vesicle transport-related. Furthermore, alcohol differentially altered proteins with certain isoforms being downregulated while others were upregulated. In summary, binge alcohol has specific effects on the maternal uterine proteome, especially those related to oxidative stress. The current study also demonstrates a great need to utilize proteomic approaches for diagnostic, mechanistic and therapeutic aspects of FASD.

Keywords

FASD; Pregnancy; Alcohol; 2-D DIGE

INTRODUCTION

Maternal alcohol consumption during pregnancy can lead to a range of deficits in the developing fetus including mental, behavioral, learning, and memory deficits that is collectively termed as Fetal Alcohol Spectrum Disorders (FASD) [1, 2]. Multiple mechanisms including direct effects of alcohol on the fetal brain as well as secondary causes

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like alcohol-induced hypoxia have been proposed [3]. However, very little is known about the role played by the mother and in particular about the maternal-fetal interface in the pathogenesis of FASD.

Numerous substantial adaptations occur at the level of the maternal uterine circulation during pregnancy including decreases in uterine vascular resistance, increased angiogenesis, and vessel remodeling [4]. The uterine endothelium plays a critical role in mediating these circulatory adaptations and is programmed during gestation to accommodate the nearly 50 fold elevation in the uterine blood flow by the third trimester of gestation [5, 6]. For example, the uterine endothelium is programmed to express elevated levels of specific proteins associated with vasodilator production including endothelial nitric oxide synthase (eNOS) [7–9], cyclooxygenase-1 [10, 11], prostacyclin synthase (PGIS) [12] etc. Although a few studies have demonstrated pregnancy-specific alcohol effects on the maternal systemic and uterine endothelium at the level of protein expression, post-translational modifications, as well as function [13, 14], there is a need for large scale proteomic studies to better understand FASD pathogenesis.

It is evident that very few proteomic studies have been reported in the field of FASD research and they were conducted in an effort to discover biomarkers [15–19]. The earliest study in this field used two-dimensional gel electrophoresis and image analysis on serum samples obtained from FASD children and discovered that no single protein uniquely identified the condition [15]. Datta and coworkers subsequently utilized MALDI-TOF/tandem MS and demonstrated decreases in amniotic fluid alpha fetoprotein using a FASD mouse model [16]. Another study by Sari et al. used in solution digestion and LC MS/MS to demonstrate differential protein expression in fetal brains of prenatally alcohol exposed mice [17]. Although these studies used label-free quantification methods that utilize variations of spectral counting procedures or the areas of distribution corresponding to the identified protein, none of them utilized routinely performed or well-established label-based techniques (e.g., ITRAQ) or 2-D DIGE to quantify the protein profiles of interest. Moreover, there is a great need to conduct FASD proteomic studies in the maternal compartment which is very critical not only from a mechanistic standpoint but also from a diagnostic and therapeutic perspective. In the present study, we utilized the 2-D DIGE method followed by MALDI-TOF MS and TOF/TOF tandem MS/MS to illustrate the alterations in alcohol-induced maternal uterine endothelial proteome. We specifically mimicked a weekend binge paradigm of drinking, a pattern common among drinking women who abuse alcohol during pregnancy [20–23].

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 [24]. The procedure for alcohol binging have been described previously [14]. Four pairs of cell lines derived four different pregnant ewes were utilized. 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) or presence 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 ovine model system [14, 25, 26], we utilized a dose of 300 mg/dl. This dose results in specific brain deficits like fetal cerebellar Purkinje cell loss [26]. Cells were exposed to a two week binge paradigm of alcohol exposure in sealed,

humidified chambers equilibrated with aqueous alcohol for three hours on three consecutive days [19, 23, 26–28], a pattern common among drinking women of child bearing age [14, 20–22]. 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.

Buffer Exchange

The protein lysates were exchanged with 2-D cell lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS) using 5 kDa molecular weight cut off spin column (Sartorius Stedim Biotech, Aubagne Cedex, France). Protein assay was carried out (Bio-Rad Protein Assay Kit, Hercules, CA) and all of the lysates were then diluted to the same protein concentration of 5 mg/ml.

Minimal CyDye labeling

2-D DIGE was run by Applied Biosystems (Hatward, CA). To 30 μg of cell lysate, 1.0 ul of diluted CyDye (1:5 diluted with DMF from 1 nmol/ul stock) was added, vortexed, and then incubated under dark on ice for 30 min. Lysine (1.0 ul of 10 mM) was added to each of the samples, vortexed and the reaction was allowed to take place under dark on ice for an additional 15 min. Cy2, Cy3 and Cy5 labeled samples were mixed followed by addition of 2X 2-D Sample buffer (8 M urea, 4% CHAPS, 20 mg/ml dithiothreitol (DTT), 2% pharmalytes and trace amount of bromophenol blue), 100 ul of destreak solution (GE Healthcare) and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and trace amount of bromophenol blue) to 250 ul for the 13 cm immobilized pH gradient (IPG) strip. The lysates were then mixed well and spun before loading the labeled samples onto the strip holder.

Isoelectric Focusing (IEF) and SDS-PAGE

After loading the labeled samples into the strip holder, 13 cm strips were laid facing down, adding 1 ml mineral oil on top of the strip. IEF was run as per manufacturer's instructions (Amersham BioSciences) under dark at 20 degree Celcius. Upon completion of the IEF, the IPG strips were incubated in the freshly made equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 10 mg/ml DTT) for 15 minutes with slow shaking. The strips were then rinsed in the freshly made equilibration buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 45 mg/ml iodoacetamide) for 10 minutes with slow shaking. The IPG strips were then rinsed once in the SDS-gel running buffer before transfer into the SDS-gel (12% SDS-gel prepared using low florescent glass plates) and sealed with 0.5% (w/v) agarose solution (in SDS-gel running buffer). The SDS-gels were run at 15 degree Celcius and until the dye front ran out of the gels.

Image scan and data analysis

Image scans were carried out immediately following the SDS-PAGE using Typhoon TRIO (Amersham BioSciences) as per the manufacturer's protocols. The scanned images were then analyzed utilizing the Image QuantTL software (GE-Healthcare), and then subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE-Healthcare). The ratio change of the differential protein expression was obtained by in-gel DeCyder software analysis.

Spot picking and digestion

The selected spots were picked up by Ettan Spot Picker (GE-Healthcare) following the DeCyder software analysis and spot picking design. The gel slice was transferred into a 500 uL microcentrifuge tube, treated with 200 uL of 50 mM ammonium bicarbonate with 50% acetonitrile and incubated for 15 minutes. The above step was repeated twice. The gel was washed twice with 400 uL water for 5 minutes and then soaked in 100% acetonitrile for 5–10 minutes. Acetonitrile was removed and then the gel was dried for 10 minutes in a speed-vac at room temperature for 30 min. The gel was subsequently rehydrated with appropriate volume of trypsin solution (10 ug/mL in 25 mM ammonium bicarbonate and 3% acetonitrile) needed to wet the entire gel surface. After 15 minutes at 37 degree Celcius, 25 mM ammonium bicarbonate was added to just cover the gel pieces and incubated at 37 degree Celcius for 16–24 hours on Thermomixer R (Eppendorf) at a speed of 550 Rpm. The supernatant was preserved. Desalting was subsequently performed using ZipTip (Zip Tip, u-C18, Tip Size: P10, Cat. No. ZTC18M960, Millipore; KIMTECH pure, #33330) 6 ul per time for higher sensitivity as follows: To enrich sample concentration, 3 ul of 50% acetonitrile and 0.1% trifluoroacetic acid was utilized to elute into the labeled sample tube. The gel spots were washed a few times then digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). The digested tryptic peptides were desalted by Zip-tip C18 (Millipore). Peptides were eluted from the Zip-tip with 0.5 ul of matrix solution (α cyano 4-hydroxycinnamic acid (5 mg/ml) in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) and spotted on the MALDI plate ABI 01-192-6-AB.

Mass Spectrometry

0.6 ul of the sample and matrix was then loaded to the MALDI-TOF slides for Peptide Mass Fingerprint (PMF) analysis, and the spot number and sample name were recorded. MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Database search

The resulting peptide mass as well as the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) to search the database of National Center for Biotechnology Information non-redundant (NCBInr). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and one missed cleavage as search parameters. Candidates with either protein score confidence interval (CI) % or Ion CI % greater than 95 were considered significant.

Immunoblotting

Post hoc validation of proteomic data was performed using Western immunoblot analysis. Protein concentration was determined using a BCA assay kit (BCA® Protein Assay, Thermo Scientific, Rockford, IL). 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 polyclonal annexin A2 (1:2000; Abcam, MA), and polyclonal β actin (1:3000; Cell Signaling, MA). After washing, the membrane was incubated with secondary goat anti-rabbit/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. The mean expression in the Alcohol group was then divided by the Control value followed by percent change calculation.

Statistics

For proteomics data, difference in the mean protein abundance between Control and Alcohol groups was analyzed using the Biological Variation Analysis Student's t test module of the DeCyder software version 6.5 (GE Health care) [29, 30]. For Western immunoblotting, Student's t test was similarly conducted between Control and Alcohol groups using Sigmaplot version 11.0. α level of significance was established *a priori* at $P < 0.05$.

RESULTS

Representative 2-D DIGE images of paired Control and Alcohol uterine endothelial samples showed a rich array of fluorescent spots (Figure 1). The green spots on the 2-D gel depict maternal uterine endothelial proteins that were downregulated by alcohol; whereas the red spots were proteins that were upregulated and the yellow spots were unaltered by binge alcohol exposure. A total of 49 fluorescent protein spots passed a test of stringent statistical criteria of a mean modulus ratio ≥ 1.5 fold [31, 32] with a $P < 0.05$ as determined by the Student's t test (ImageQuant and DeCyder softwares; Figure 2). A total of 30 proteins were significantly ($P < 0.05$) decreased in response to chronic binge alcohol whereas the remaining 19 were increased ($P < 0.05$). These altered proteins formed a rich set associated with numerous biological categories including protein translation (e.g., elongation factor, TRNA synthetase, 28S ribsosomal protein S22), cell structure (e.g., tubulin isoforms, myosin light chain isoform, destrin), ion channels (e.g., chloride ion channel isoforms), extracellular matrix (e.g., connective tissue growth factor), enzymes (e.g., prolyl hydroxylase, glutathione transferase, transglutaminase) and cell-cell adhesion (e.g., annexin).

Gene-enrichment and functional annotation cluster analysis revealed five specific classes including glutathione S transferase ($P < 0.05$), thioredoxin ($P < 0.05$), vesicle transport-related ($P < 0.05$), ion channels (not significant), and nucleotide binding-related (not significant; Figure 3). Of these, glutathione S transferase had the highest overall enrichment score of 2.95. This was followed by the thioredoxin with an overall enrichment score of 2.87. The vesicle transport proteins also exhibited a significant overall enrichment score of 1.63.

Interestingly, numerous alterations in post-translational modifications were noted; different isoforms of the same protein were detected as distinct spots in the 2-D DIGE gel. To elaborate this finding, Figure 4 depicts the two detected isoforms of the protein chloride intracellular channel 1; the protein was altered such that one isoform (spot 21) was decreased whereas the other (spot 22) was increased in response to chronic binge alcohol. Analysis of the surface areas of spot 22 are depicted in Figure 4 demonstrating the effect of alcohol on multi-site post-translational modifications.

Post hoc validation was performed for the protein annexin A2, specifically chosen because the sheep antibody was commercially available (Figure 5). As illustrated, β actin showed no alteration and is seen as a bright yellow spot in the 2-D gel. This observation was validated by immunoblotting. Two isoforms of annexin were detected by 2-D DIGE/mass

spectrometry; one isoform exhibited a 149% decrease (−2.49 fold of Control; $P < 0.05$) and the other a 59% increase (+1.59 fold of Control; $P < 0.05$) in response to binge alcohol. However, because the two isoforms change in opposite direction with alcohol treatment, epitope non-specific immunoblotting demonstrated no alteration ($P = 0.432$) in annexin A2 levels.

DISCUSSION

To our knowledge this is the first study ever to utilize quantitative 2-D DIGE for proteomic analysis on any cell in the field of FASD. The major findings that can be gleaned from this study are: 1. Chronic binge-like alcohol substantially affects the maternal uterine vascular compartment at the level of the endothelial proteome; 2. Gene-enrichment and functional annotation cluster analysis demonstrated significant enrichment in three classes: glutathione S transferase P, thioredoxin, and those related to vesicle transport; 3. In addition to alterations in whole protein expression levels, alcohol produces post-translational modifications such that specific isoforms are downregulated while others are upregulated; 4. Despite the advantages of the routinely performed epitope-nonspecific Western immunoblotting, our data demonstrate a great need to utilize proteomic methodologies for more as yet unrealized comprehensive protein functional analyses.

The present data set demonstrates that chronic binge-like alcohol has direct and considerable effects on the maternal uterine vascular compartment specifically at the level of the entire endothelial proteome. In pursuit of discovering the mechanisms underlying FASD pathogenesis, most studies have focused on the direct local effects of alcohol on the developing fetal brain [1, 3]. However, it has also been suggested that alcohol may produce the spectrum of deficits in the developing fetus via secondary mechanisms in addition to the widely studied direct effects [3]. These secondary mechanisms are attributed to changes in maternal gestational physiology, specifically at the level of the utero-placental interface, and possibly even parturition [28, 33]. Only a few studies have focused on pregnancy-specific alcohol effects on the maternal systemic and uterine endothelium at the level of protein expression, post-translational modifications, as well as function [13, 14]. Specifically, at the level of the maternal uterine artery endothelial cells, alcohol is known to impact the nitric oxide system, a regulator of uterine vascular adaptations during pregnancy via decreases in the expression and activity of the rate limiting enzyme eNOS [14]. Further, alcohol produces negative effects on maternal uterine angiogenesis [14], uterine blood flow [34], as well as feto-placental blood low [34, 35]. However, in contrast to these findings one study surprisingly reported increases in uterine blood flow in response to an alcohol infusion divided into four intermittent doses [36]. Though it is clear that alcohol impacts the uterine vasculature from these studies, the differences in the outcomes may stem from dose, timing, as well as the pattern (acute, chronic, intermittent) of alcohol exposure [22] or an adaptive response by changing overall downstream intrinsic uterine vascular resistance or possible extrinsic perfusion pressure [37]. In the current study, we did not identify eNOS or PGIS. This was expected as these are compartmentalized proteins and are localized in specific sub-cellular domains termed as caveolar lipid rafts and their analyses require uterine artery endothelial caveolar enrichment. In summary, the current data is quite consistent with these reports [14, 34, 38, 39] and further extends these concepts by demonstrating that alcohol directly alters the uterine artery endothelial proteome in the pregnant state. Thus, these data point to an important role for the uterine vasculature in FASD pathogenesis.

Gene-enrichment and functional annotation cluster analysis showed significant enrichment ($P < 0.05$) in three groups: glutathione S transferase, thioredoxin, and those related to vesicle transport. Gluthione S transferase is an integral component of the endogenous detoxification defense strategy which allows the cells or the organism to adapt to environments of

oxidative stress and inflammation [40]. The present data also demonstrates for the first time in the uterine endothelium the alteration of the specific protein glutathione S transferase P, a placental isoform that is localized in the feto-placental unit with substantial expression in the cytosol of the chorionic villi [41, 42]. The second annotation cluster with a significant enrichment score was thioredoxin, a major intracellular redox balance regulating family comprising of many anti-oxidative proteins and a biomarker for oxidative stress [43]. This is a significant finding as numerous reports have cited alcohol-induced oxidative stress as primary mechanism for mediating fetal brain damage [3]. The present observations now extend the existing knowledge for the first time to the maternal compartment and thus may have important implications for alcohol-induced alterations in uterine vascular adaptations during pregnancy. Supporting our findings, an association between oxidative stress and reductions in uterine blood flow have been reported in compromised pregnancies such as preeclampsia [44]. Further, the maternal intralobular antioxidant enzyme activity also directly reflects the pattern of maternal blood flow [45]. Finally, the annotation cluster associated with vesicle transport exhibited a high enrichment score. This supports previously published data on effects of alcohol on sub-cellular protein dynamics [46].

Multiple isoforms of the same protein were altered such that certain isoforms were upregulated whereas others were downregulated. This is consistent with previously reported data that alcohol differentially alters the multi-site phosphorylation of enzymes [14]. For further validation, we specifically performed immunoblotting for annexin A2 for which the antibody was commercially available for sheep. Two isoforms of annexin A2 were detected by proteomics; one isoform was upregulated whereas the other was decreased. However, the commercially available epitope-independent annexin A2 ovine antibody detected no difference between groups. This may be because the two isoforms changed in opposite direction with alcohol treatment. These data also suggest the need for more proteomic studies in addition to the routinely performed non-epitope specific Western immunoblotting to draw elaborate conclusions on protein function.

There is a great need for conducting proteomic studies to comprehend the mechanistic perspectives underlying the pathogenesis of FASD [15–19]. The current study is the first in the FASD research area to utilize quantitative 2-D DIGE methodology for this purpose. We specifically wanted to exploit the advantages of the 2-D DIGE technology, the only method that can be used for simultaneous quantitation of control and treated complex biological protein mixtures with minimum technical variability [47]. Although outside the FASD research area, both label-based and label-free mechanisms have been used to quantify proteomic profiles, it is suggested that there still exists several challenges in deriving meaningful explanations from these data to gain a proper understanding of the physiologic phenomena [48].

We conclude that chronic binge alcohol has effects at the level of the mother, specifically the maternal uterine compartment. Therefore, in addition to the direct effects at the level of the fetal brain, therapeutic strategies to prevent and/or ameliorate FASD must take the mother, the uterus, the placenta and parturition into consideration. Moreover, there is a great need for conducting proteomic studies to not only explore biomarkers, but also for understanding functional mechanistic perspectives on the course of disease pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Riley EP, McGee CL. Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. Exp Biol Med (Maywood). 2005; 230:357–65. [PubMed: 15956765]
2. Sokol RJ, Delaney-Black V, Nordstrom B. Fetal alcohol spectrum disorder. Jama. 2003; 290:2996–9. [PubMed: 14665662]
3. Goodlett CR, Horn KH, Zhou FC. Alcohol teratogenesis: mechanisms of damage and strategies for intervention. Exp Biol Med (Maywood). 2005; 230:394–406. [PubMed: 15956769]
4. Osol G, Mandala M. Maternal uterine vascular remodeling during pregnancy. Physiology (Bethesda). 2009; 24:58–71. [PubMed: 19196652]
5. Rosenfeld CR. Distribution of cardiac output in ovine pregnancy. Am J Physiol. 1977; 232:H231–5. [PubMed: 842676]
6. Magness RR, Phernetton TM, Gibson TC, Chen DB. Uterine blood flow responses to ICI 182 780 in ovariectomized oestradiol-17beta-treated, intact follicular and pregnant sheep. J Physiol. 2005; 565:71–83. [PubMed: 15774510]
7. Yi FX, Magness RR, Bird IM. Simultaneous imaging of [Ca2+]i and intracellular NO production in freshly isolated uterine artery endothelial cells: effects of ovarian cycle and pregnancy. Am J Physiol Regul Integr Comp Physiol. 2005; 288:R140–8. [PubMed: 15297265]
8. Magness RR, Shaw CE, Phernetton TM, Zheng J, Bird IM. Endothelial vasodilator production by uterine and systemic arteries. II. Pregnancy effects on NO synthase expression. Am J Physiol. 1997; 272:H1730–40. [PubMed: 9139957]
9. Magness RR, Sullivan JA, Li Y, Phernetton TM, Bird IM. Endothelial vasodilator production by uterine and systemic arteries. VI. Ovarian and pregnancy effects on eNOS and NO(x). Am J Physiol Heart Circ Physiol. 2001; 280:H1692–8. [PubMed: 11247781]
10. Janowiak MA, Magness RR, Habermehl DA, Bird IM. Pregnancy increases ovine uterine artery endothelial cyclooxygenase-1 expression. Endocrinology. 1998; 139:765–71. [PubMed: 9449651]
11. Habermehl DA, Janowiak MA, Vagnoni KE, Bird IM, Magness RR. Endothelial vasodilator production by uterine and systemic arteries. IV. Cyclooxygenase isoform expression during the ovarian cycle and pregnancy in sheep. Biol Reprod. 2000; 62:781–8. [PubMed: 10684824]
12. Magness RR, Shideman CR, Habermehl DA, Sullivan JA, Bird IM. Endothelial vasodilator production by uterine and systemic arteries. V. Effects of ovariectomy, the ovarian cycle, and pregnancy on prostacyclin synthase expression. Prostaglandins Other Lipid Mediat. 2000; 60:103–18. [PubMed: 10751641]
13. Cook JL, Zhang Y, Davidge ST. Vascular function in alcohol-treated pregnant and nonpregnant mice. Am J Physiol Regul Integr Comp Physiol. 2001; 281:R1449–55. [PubMed: 11641115]
14. Ramadoss J, Jobe SO, Magness RR. 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. Alcohol Clin Exp Res. 2011; 35 In Press.
15. Robinson MK, Myrick JE, Henderson LO, Coles CD, Powell MK, Orr GA, et al. Two-dimensional protein electrophoresis and multiple hypothesis testing to detect potential serum protein biomarkers in children with fetal alcohol syndrome. Electrophoresis. 1995; 16:1176–83. [PubMed: 7498162]
16. Datta S, Turner D, Singh R, Ruest LB, Pierce WM Jr, Knudsen TB. Fetal alcohol syndrome (FAS) in C57BL/6 mice detected through proteomics screening of the amniotic fluid. Birth Defects Res A Clin Mol Teratol. 2008; 82:177–86. [PubMed: 18240165]
17. Sari Y, Zhang M, Mechref Y. Differential expression of proteins in fetal brains of alcohol-treated prenatally C57BL/6 mice: a proteomic investigation. Electrophoresis. 2010; 31:483–96. [PubMed: 20119957]

18. Bearer CF, Stoler JM, Cook JD, Carpenter SJ. Biomarkers of alcohol use in pregnancy. Alcohol Res Health. 2004; 28:38–43. [PubMed: 19006990]
19. Ramadoss J, Liao WX, Chen DB, Magness RR. High-throughput caveolar proteomic signature profile for maternal binge alcohol consumption. Alcohol. 2010; 44:691–7. [PubMed: 20053519]
20. Gladstone J, Nulman I, Koren G. Reproductive risks of binge drinking during pregnancy. Reprod Toxicol. 1996; 10:3–13. [PubMed: 8998383]
21. Caetano R, Ramisetty-Mikler S, Floyd LR, McGrath C. The epidemiology of drinking among women of child-bearing age. Alcohol Clin Exp Res. 2006; 30:1023–30. [PubMed: 16737461]
22. Maier SE, West JR. Drinking patterns and alcohol-related birth defects. Alcohol Res Health. 2001; 25:168–74. [PubMed: 11810954]
23. Ramadoss J, Lunde ER, Pina KB, Chen WJ, Cudd TA. All three trimester binge alcohol exposure causes fetal cerebellar purkinje cell loss in the presence of maternal hypercapnea, acidemia, and normoxemia: ovine model. Alcohol Clin Exp Res. 2007; 31:1252–8. [PubMed: 17511745]
24. Bird IM, Sullivan JA, Di T, Cale JM, Zhang L, Zheng J, et al. Pregnancy-dependent changes in cell signaling underlie changes in differential control of vasodilator production in uterine artery endothelial cells. Endocrinology. 2000; 141:1107–17. [PubMed: 10698187]
25. Cudd TA, Chen WJ, Parnell SE, West JR. Third trimester binge ethanol exposure results in fetal hypercapnea and acidemia but not hypoxemia in pregnant sheep. Alcohol Clin Exp Res. 2001; 25:269–76. [PubMed: 11236842]
26. West JR, Parnell SE, Chen WJ, Cudd TA. Alcohol-mediated Purkinje cell loss in the absence of hypoxemia during the third trimester in an ovine model system. Alcohol Clin Exp Res. 2001; 25:1051–7. [PubMed: 11505032]
27. Eysseric H, Gonthier B, Soubeyran A, Bessard G, Saxod R, Barret L. There is not simple method to maintain a constant ethanol concentration in long-term cell culture: keys to a solution applied to the survey of astrocytic ethanol absorption. Alcohol. 1997; 14:111–5. [PubMed: 9085710]
28. Ramadoss J, Lunde ER, Chen WJ, West JR, Cudd TA. Temporal vulnerability of fetal cerebellar Purkinje cells to chronic binge alcohol exposure: ovine model. Alcohol Clin Exp Res. 2007; 31:1738–45. [PubMed: 17681031]
29. Alfonso P, Nunez A, Madoz-Gurpide J, Lombardia L, Sanchez L, Casal JI. Proteomic expression analysis of colorectal cancer by two-dimensional differential gel electrophoresis. Proteomics. 2005; 5:2602–11. [PubMed: 15924290]
30. Damodaran S, Dlugos CA, Wood TD, Rabin RA. Effects of chronic ethanol administration on brain protein levels: a proteomic investigation using 2-D DIGE system. Eur J Pharmacol. 2006; 547:75–82. [PubMed: 16978605]
31. Felty Q. Proteomic 2D DIGE profiling of human vascular endothelial cells exposed to environmentally relevant concentration of endocrine disruptor PCB153 and physiological concentration of 17beta-estradiol. Cell Biol Toxicol. 2011; 27:49–68. [PubMed: 20623170]
32. Brechlin P, Jahn O, Steinacker P, Cepek L, Kratzin H, Lehnert S, et al. Cerebrospinal fluid-optimized two-dimensional difference gel electrophoresis (2-D DIGE) facilitates the differential diagnosis of Creutzfeldt-Jakob disease. Proteomics. 2008; 8:4357–66. [PubMed: 18814332]
33. Cudd TA. Animal model systems for the study of alcohol teratology. Exp Biol Med (Maywood). 2005; 230:389–93. [PubMed: 15956768]
34. Falconer J. The effect of maternal ethanol infusion on placental blood flow and fetal glucose metabolism in sheep. Alcohol Alcohol. 1990; 25:413–6. [PubMed: 2222575]
35. Jones PJ, Leichter J, Lee M. Placental blood flow in rats fed alcohol before and during gestation. Life Sci. 1981; 29:1153–9. [PubMed: 7289779]
36. Reynolds JD, Penning DH, Dexter F, Atkins B, Hrdy J, Poduska D, et al. Ethanol increases uterine blood flow and fetal arterial blood oxygen tension in the near-term pregnant ewe. Alcohol. 1996; 13:251–6. [PubMed: 8734839]
37. Magness, RR. The Endocrinology of Pregnancy Bazer. Humana Press; 1998. Maternal cardiovascular and other physiologic responses to the endocrinology of pregnancy; p. 507-39.
38. Rosenberg MJ, Wolff CR, El-Emawy A, Staples MC, Perrone-Bizzozero NI, Savage DD. Effects of moderate drinking during pregnancy on placental gene expression. Alcohol. 2010; 44:673–90. [PubMed: 20053520]

39. Shukla PK, Sittig LJ, Ullmann TM, Redei EE. Candidate Placental Biomarkers for Intrauterine Alcohol Exposure. Alcohol Clin Exp Res. 2010
40. Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annu Rev Pharmacol Toxicol. 2005; 45:51–88. [PubMed: 15822171]
41. Bearer CF. How are children different from adults? Environ Health Perspect. 1995; 103 (Suppl 6): 7–12. [PubMed: 8549494]
42. Polidoro G, Di Ilio C, Del Boccio G, Zulli P, Federici G. Glutathione S-transferase activity in human placenta. Biochem Pharmacol. 1980; 29:1677–80. [PubMed: 7406897]
43. Kobayashi-Miura M, Shioji K, Hoshino Y, Masutani H, Nakamura H, Yodoi J. Oxygen sensing and redox signaling: the role of thioredoxin in embryonic development and cardiac diseases. Am J Physiol Heart Circ Physiol. 2007; 292:H2040–50. [PubMed: 17293486]
44. Roberts JM, Gammill HS. Preeclampsia: recent insights. Hypertension. 2005; 46:1243–9. [PubMed: 16230510]
45. Hempstock J, Bao YP, Bar-Issac M, Segaren N, Watson AL, Charnock-Jones DS, et al. Intralobular differences in antioxidant enzyme expression and activity reflect the pattern of maternal arterial bloodflow within the human placenta. Placenta. 2003; 24:517–23. [PubMed: 12744928]
46. Mao H, Diehl AM, Li YX. Sonic hedgehog ligand partners with caveolin-1 for intracellular transport. Lab Invest. 2009; 89:290–300. [PubMed: 19139721]
47. Hoorn EJ, Hoffert JD, Knepper MA. The application of DIGE-based proteomics to renal physiology. Nephron Physiol. 2006; 104:p61–72. [PubMed: 16721037]
48. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem. 2007; 389:1017–31. [PubMed: 17668192]

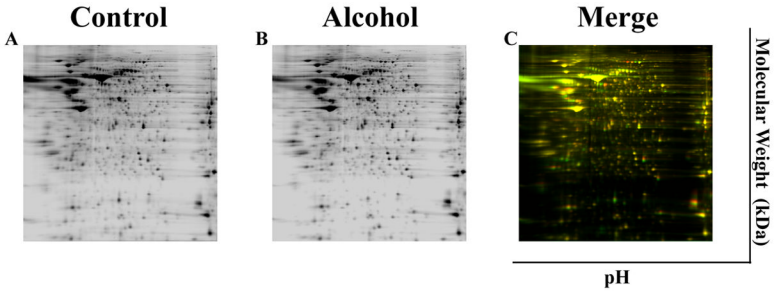

Figure 1.

Representative 2-D DIGE images of paired Control and Alcohol uterine endothelial samples depicting a rich array of fluorescent spots. Equal amounts of Cy2 (standard with equally mixed samples), Cy3 (Control), and Cy5 (alcohol) -labeled samples were mixed and then separated on analytical 2-D DIGE. (A, B) The black and white images depict fluorescent signals derived from the red and green channels. (C) The green spots on the 2-D gel depict maternal uterine endothelial proteins that were downregulated by alcohol; whereas the red spots were proteins that were upregulated and the yellow spots were unaltered by binge alcohol exposure.

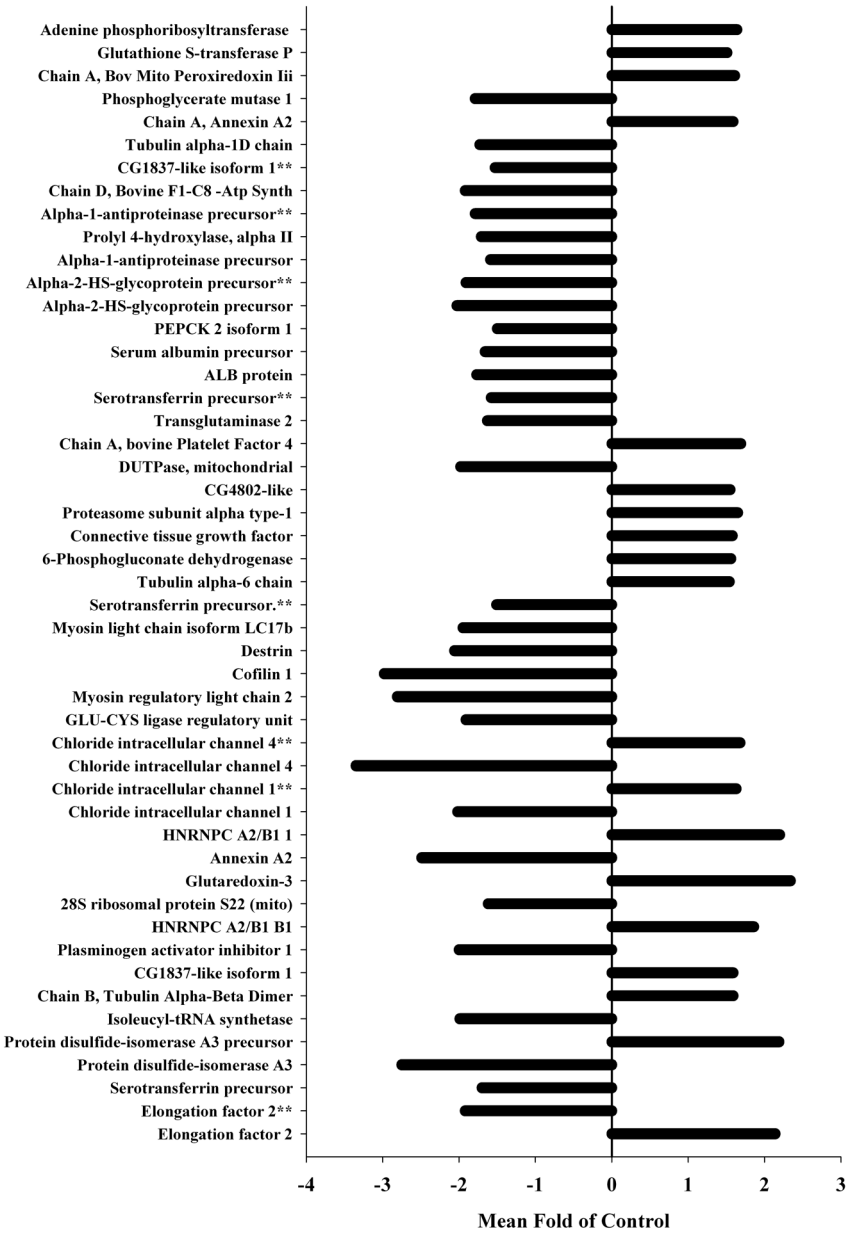

Figure 2.

A total of 49 fluorescent protein spots passed a test of stringent statistical criteria of a mean modulus ratio ≥ 1.5 fold with a $P < 0.05$ as determined by the Student's t test (ImageQuant and DeCyder softwares). 30 proteins were significantly decreased in response to chronic binge alcohol whereas the remaining 19 were increased. These altered proteins formed a rich set associated with numerous biological categories including those related to protein translation, cell structure, ion channels, extracellular matrix, enzymes, and cell-cell adhesion. ** represents a different isoform of the same protein.

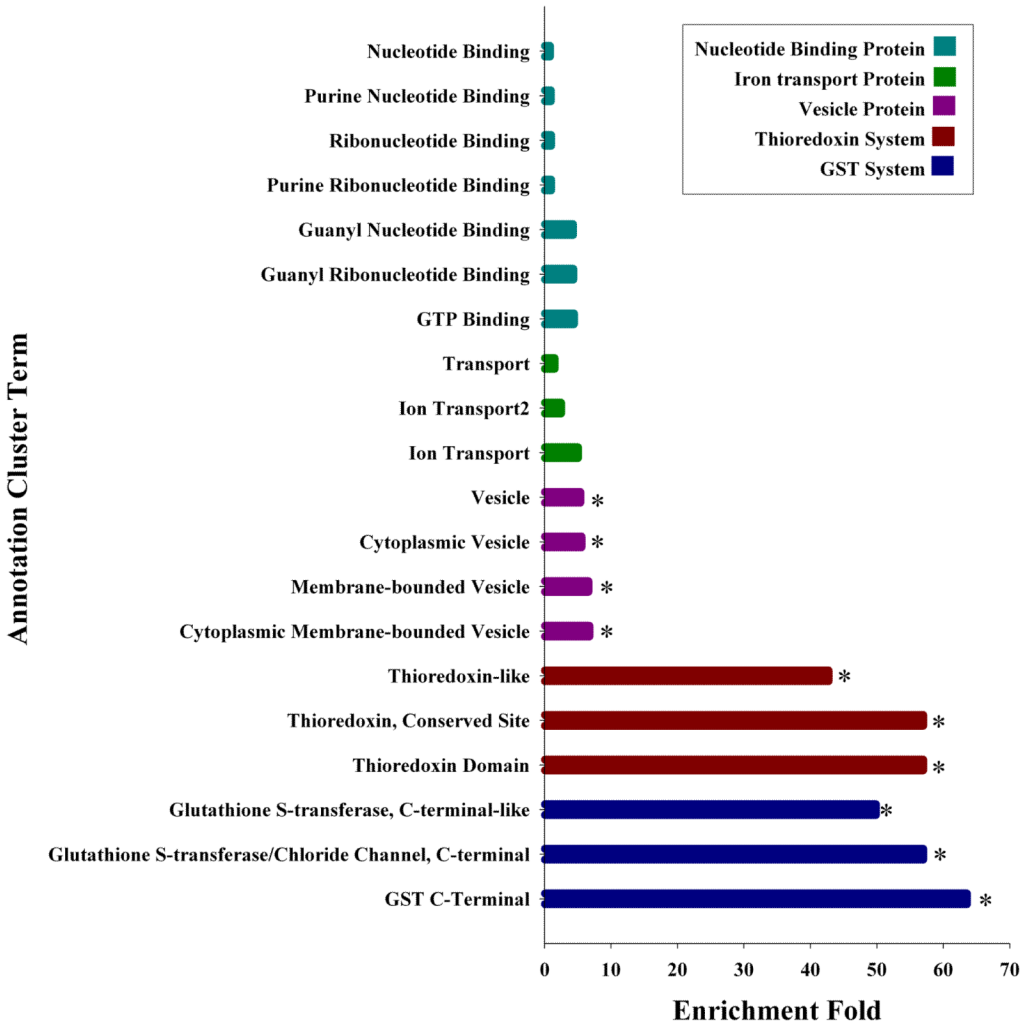

Figure 3.

Gene-enrichment and functional annotation cluster analysis revealed five specific classes: glutathione S transferase ($P < 0.05$), thioredoxin ($P < 0.05$), vesicle transport-related ($P < 0.05$), ion channels (not significant), and nucleotide binding-related (not significant). The y axis depicts the term in the annotation cluster and the x axis represents the fold enriched relative to the percent of genes in the whole genome; GST, Glutathione S Transferase.

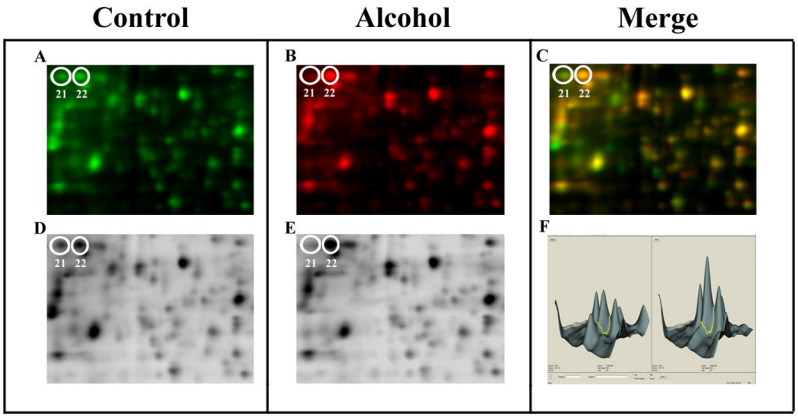

Figure 4.

Illustration of binge alcohol-induced differential alteration of posttranslational modifications. (A–E) The circled spots (chloride intracellular channel 1) were altered such that one isoform (spot 21) was decreased whereas the other (spot 22) was increased in response to binge alcohol. (F) Analysis of the surface area of spot 22 is depicted; Left, Control; Right, Alcohol.

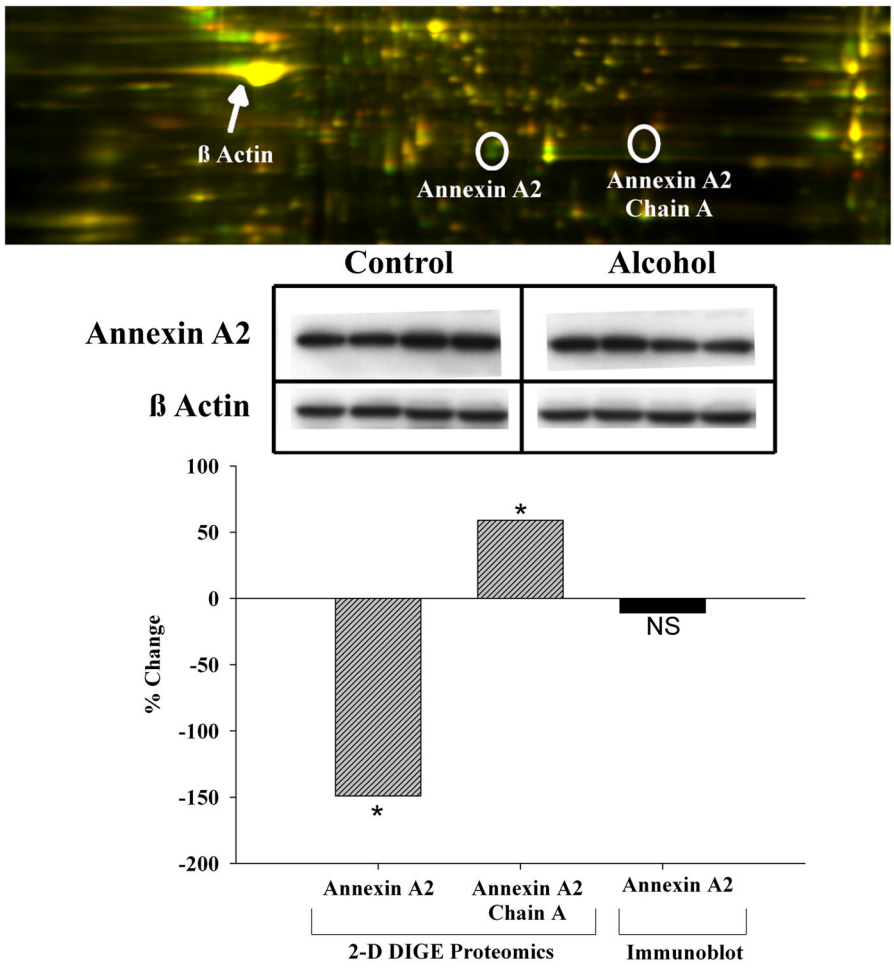

Figure 5.

Post hoc validation was performed for the protein annexin A2, for which the sheep antibody was commercially available. β actin (identified based on isoelectric point, molecular weight, and previous studies) showed no alteration and is seen as a bright yellow spot in the gel and is validated by immunoblotting. Two isoforms of annexin were detected by 2-D DIGE/mass spectrometry: one isoform exhibited a 149% decrease (−2.49 fold of Control; $P < 0.05$) and the other a 59% increase (+1.59 fold of Control; $P < 0.05$) in response to binge alcohol. However, non-specific immunoblotting demonstrated no alteration ($P = 0.432$) in annexin A2 expression levels; *, $P < 0.05$; NS, Not Significant.