Proteomic Analysis of Baboon Cerebral Artery Reveals Potential Pathways of Damage by Prenatal Alcohol Exposure

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In Brief
Proteome analysis was performed to determine whether fetal alcohol exposure during mid-pregnancy would evoke changes in protein profile of fetal cerebral artery in baboons. We detected that levels of 238 proteins differed significantly between control and alcohol-exposed fetuses. Proteins of metabolic pathways represented one of the major targets of alcohol. The differences were detected near term, long after alcohol exposure took place. Our findings point at novel targets of alcohol within developing brain vessels.

Highlights
• We studied mid-pregnancy alcohol exposure and baboon fetal cerebral artery.
• 238 proteins differed between control and alcohol-exposed fetuses near-term.
• Proteins of metabolic pathways represented one of the major targets of alcohol.
• Alcohol effect on the development of fetal brain vessels is persistent.
Proteomic Analysis of Baboon Cerebral Artery Reveals Potential Pathways of Damage by Prenatal Alcohol Exposure* [S]

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Alcohol is one of the most widely abused substances in the world. Alcohol consumption by pregnant women often results in an array of fetal developmental abnormalities, but the damage to the fetus by alcohol remains poorly understood. The limited knowledge regarding the molecular targets of alcohol in the developing fetus constitutes one of the major obstacles in developing effective pharmacological interventions that could prevent fetal damage after alcohol consumption by pregnant women. The fetal cerebral artery is emerging as an important mediator of fetal cerebral damage by maternal alcohol drinking. In the present work, we conduct proteomics analysis of cerebral (basilar) artery lysates of near-term fetal baboons to search for protein targets of fetal alcohol exposure. Our study demonstrates that 3 episodes of binge alcohol exposure during the second trimester-equivalent of human pregnancy are sufficient to render profound changes in fetal cerebral artery proteome. These changes persisted, as they were detected in near-term fetuses. In particular, the relative abundance of 238 proteins differed significantly between control and alcohol-exposed fetuses. Enrichment analysis pointed at the group of metabolic activity proteins as a major class targeted by alcohol. Western blotting confirmed upregulation of the aldehyde dehydrogenase 6 family member A1 (ALDH6A1) in cerebral artery lysates from alcohol-exposed fetuses. This upregulation translated to greater ALDH activity of cerebral artery lysate of near-term fetuses following prenatal alcohol exposure when compared with controls. Molecular & Cellular Proteomics 18: 294–307, 2019. DOI: 10.1074/mcp.RA118.001047.

The contribution of proteomics to the management of vascular and neurodegenerative disorders has been increasingly recognized (1–2). The proteome of cerebral arteries has been assessed in detail in a mouse model (3). Comparative proteomic analysis has revealed changes in protein networks following experimental subarachnoid hemorrhage of rat cerebral arteries (4) and in ischemic brain of permanent middle cerebral artery occlusion in a mouse model (5). In addition, changes in brain vessel proteome were described in Alzheimer’s disease mice (6). A growing body of evidence strongly suggests that the cerebral circulation is a critical target of prenatal alcohol exposure (7–11). The current work represents the first documentation of proteome changes in fetal cerebral arteries following prenatal alcohol exposure.

Alcohol (ethanol) is one of the most widely consumed psychoactive drugs in the world (12). Despite major research and education efforts describing the deleterious effects of excessive alcohol consumption on human health, alcohol continues to be consumed by pregnant women. Studies in the United States, Canada, Australia and New Zealand report that 5–50% of pregnant women engaged in alcohol consumption at some time during pregnancy (13–16).

As documented by numerous studies, binge alcohol consumption, with sharp and high peaks in maternal blood alcohol concentration (BAC) above 80 mg/dL, produces the most negative effects on the developing fetus (17–19). As a result, prenatal alcohol exposure (PAE) can lead to a wide range of cognitive and morphological abnormalities, including fetal alcohol spectrum disorders (FASD), with the most severe cases conforming to what has been defined as fetal alcohol syndrome (FAS) (20–21). In countries with the highest percentage of alcohol consumption during pregnancy, FAS/FASD prevalence reaches 6%, with the global prevalence of FASD among children and youth being estimated at 0.77% (22–26).

Currently, there is no readily available cure for the lifelong consequences of PAE (27–28). One of the reasons is the lack of mechanistic understanding of FAS/FASD pathology. Individualization of specific and relevant ethanol targets is difficult because ethanol is a rather promiscuous ligand that targets multiple molecular targets, organs and systems. This low specificity is primarily related to the chemical simplicity of the ethanol molecule (29–31). However, the brain constitutes the most severely affected organ, exhibiting both structural and functional abnormalities in response to PAE (31–32).
Recently, the effects of alcohol on fetal cerebrovascular function started receiving increasing attention, as neuronal survival relies on an adequate blood supply (33). Studies in several species consistently implicate increased fetal cerebral perfusion following maternal alcohol intoxication (8–9, 11, 34). Alcohol-driven alteration in cerebrovascular function is associated with long-lasting effects on fetal development. Our recent study utilizing Doppler sonography on pregnant baboons shows that alterations in fetal cerebral artery indices in instances of maternal alcohol consumption during the period equivalent to the second trimester of human pregnancy precedes morphometric developmental delays in alcohol-exposed fetuses (11). In an ovine model, larger alterations in fetal cerebral blood flow in the presence of alcohol were linked to the severity of subsequent neuronal loss (7). Moreover, effects of prenatal alcohol exposure on cerebral artery function can be detected long after birth (8, 35). Thus, there is no doubt that there are long-lasting consequences of prenatal alcohol exposure on fetal cerebral artery at the functional level. However, the molecular targets of ethanol action on fetal cerebral arteries are largely unknown.

In the current manuscript, we applied a proteomics analysis to investigate the protein profile of basilar arteries of near-term fetal baboons that had been exposed to alcohol during the second trimester-equivalent of pregnancy and compare this profile to a proteome of alcohol-naïve fetuses. Despite the high prevalence of alcohol consumption among pregnant women, studying the mechanisms underlying alcohol-induced changes in the development of human fetuses is unfeasible for several reasons. First, intended exposure of human fetuses to alcohol is ethically unacceptable. Second, even with access to a maternal population, it is nearly impossible to standardize patterns and amounts of alcohol consumption during pregnancy. Much of the available information regarding alcohol consumption during pregnancy is obtained based on maternal self-reports (36–38). Although this noninvasive approach is found to be valid and reliable (39–40), accuracy may require improvement because of the subjectivity of the reporting, including under-reporting of drinking during pregnancy (41–42). Finally, there is a multitude of confounding factors in a human population, including genetic background, maternal health history, diet, multisubstance abuse, and socioeconomic status (43).

Our choice of baboons was dictated by the fact that these nonhuman primates shared key reproductive features and fetal development milestones with humans (44–48). In our experimental paradigm, each episode of alcohol exposure resulted in 80 mg/dL maternal BAC within 60 min of drink infusion into pregnant dams via gastric gavage (10). Our study demonstrates that only three episodes of binge alcohol exposure during second trimester-equivalent of pregnancy render wide-ranging changes in the basilar artery protein profile of near-term fetuses.

MATERIALS AND METHODS

Ethics and Study Approval

The care of animals and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center, which is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACi)-accredited institution. The current manuscript was prepared following the Animals in Research: Reporting in Vivo Experiments (ARRIVE) guidelines (49).

Animal Subjects

Baboons were received from the University of Oklahoma Primate Research Center. The gestational age of candidate baboons for the study was determined by this Center based on female baboon cycles and confirmed with ultrasound examination. Before reaching 80 days of gestation, baboon dams were transported to UTHSC and given 10 days to acclimate to the new environment. Dams were alcohol-naïve but had been used in other research studies. Baboons were housed at UTHSC as detailed in our recent work (10). Briefly, dams were singly housed in standard baboon cages, with visual and audio access to each other. A maximum of four baboons (cages) were housed per room, on a 12-hour light/dark cycle (lights on at 6:00 am). Baboons were fed twice a day, each feeding consisting of the High Protein Monkey Diet (~15 biscuits per meal, 21 kcal/biscuit) to sustain a baboon’s weight gain as expected throughout pregnancy. Each feeding was also supplemented by two pieces of fresh fruits or vegetables and two tablespoons of peanut butter. Drinking water was available ad libitum. Facilities were maintained in accordance with the USDA and AAALACi standards. Species-appropriate toys, video presentations, and human interactions were provided to dams daily as a means of environmental and social enrichment. Housing conditions were consistent throughout the study.

Confirmation of Gestational Age and Alcohol Infusion Procedure

Dams were subjected to either alcohol or control infusion procedures at 90, 100, and 110 days of gestation (full term in baboons is 175–185 days). Ninety to 110 days of baboon gestation correspond to the mid-pregnancy during which fetal baboon cerebral arteries had documented acute response to alcohol (10). On the day of the first procedure (90 ± 5 days of gestation), animals were anesthetized (see below) and gestational age was once again confirmed by sonography using SonoAce R3 by Samsung (Seoul, South Korea). Fetal abdominal circumference, head circumference and femur length were measured in millimeters and compared with commonly accepted fetal baboon growth curve variables to verify the gestational age. The study involved a total of seven Papio hamadryas anubis dams (ages 7–15 yrs; 90 ± 5 days of gestation) carrying single fetuses. Dams were randomly assigned to the experimental groups: three dams received control infusions, whereas four dams received alcohol-containing infusions. For the experimental group, the infusion contained 1.8 g/kg ethanol (ultrapure, 200 proof; American Bioanalytical, Natick, MA) diluted in reverse osmosis purified drinking water. This alcohol dose rendered on average 80 mg/dL alcohol in maternal blood and 63 mg/dL alcohol in amniotic fluid (10). The control group of animals received an isocaloric solution containing orange-flavored Tang® powder (Kraft Foods; Northfield, IL). In both cases, the total volume of the drink was equal to 200 ml. Before each infusion procedure, the animals were fasted for 12 h. On the day of the infusion, the animal sedation was induced by a single injection of ketamine hydrochloride.
(Ketaset, 10 mg/kg IM). Throughout the infusion, anesthesia was maintained with isoflurane (1.5–2.0%) in oxygen. Animal monitoring of vital signs and depth of anesthesia consisted of electrocardiography, pulse oximetry, capnography, and noninvasive blood pressure and temperature measurements. A rectal suppository of indomethacin (25 mg) was used as preterm labor prophylaxis during the procedure. An orogastric tube was introduced into the stomach and the infusion was administered over 10 min. Both groups of animals received a single injection of carpfen (Rimadyl, 4.4 mg/kg IM) aimed to alleviate symptoms of hangover following alcohol drinking. Cesarean deliveries were performed at 165 ± 5 days of gestation. This gestational age represents near-term, as term pregnancy in baboons is described as 175–185 days of gestation (50). During c-section, fetuses were euthanized by exsanguination while still under maternal anesthesia and subsequently administered Euthasol (0.5 ml per fetus IV). Two female and 1 male fetuses were obtained in the control group, and 4 female fetuses were obtained in the alcohol-exposed group. Fetal brain tissue was harvested for basilar artery dissection.

**Tissue Lysate Preparation**

Fetal baboon basilar arteries were rinsed with phosphate-buffered saline, then leftovers of neuronal tissue were removed under the microscope. Artery lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer of the following composition: 50 mM NaCl, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM tris, pH = 8.0. Protease and phosphatase inhibitor mixture (78441, Thermo Fisher) was added to RIPA buffer before use. The tissue was homogenized using BeadBug homogenizer (MIDSCI) according to manufacturer’s instructions. After 20 mins of incubation on ice, lysate was centrifuged at 825 x g for 10 mins in Eppendorf benchtop centrifuge. The supernatants were collected, and protein levels were measured using BCA Protein Assay Kit (23225, Thermo Fisher) following manufacturer’s protocol—8 step fractions (consecutively eluted with 10.0, 12.5, 15.0, 17.5, 20.0, 22.5, 25.0, and 50.0% acetonitrile) were collected. The collected peptide fractions were vacuum dried.

**LC-MS**—Each dried peptide fraction (~11 µg) was re-dissolved in 100 µl of loading buffer (3% acetonitrile, 0.05% TFA), and 5 µl (0.55 µg) was analyzed by LC-MS for peptide/protein identification and quantification. Raw MS data were acquired on an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher) operating in line with an Ultimate 3000 RSLC nano UHPLS system (Thermo Fisher). The peptides were trapped on an Acclaim PepMap 100 nanoViper column (75 µm x 20 mm, Thermo Fisher) at 5 µl/min flow rate. The trapped peptides were separated on an Acclaim PepMap RSLC nanoViper column (75 µm x 500 mm, C-18, 2 µm, 100 Å, Thermo Fisher) at 300 nl/min flow rate and 40 °C column temperature using water and acetonitrile with 0.1% formic acid as solvents A and B, respectively. The following multipoint linear gradient was applied: 3% B at 0–4 min, 5% B at 5 min, 23% B at 110 min, 30% B at 120 min, 90% B at 123–133 min, and 3% B at 136–160 min. The data-dependent MS analysis was performed on ions with charge state 2–6; MS3 Synchronous Precursor Selection (SPS) method was used with 3 s cycles and the following scan parameters. MS (full) scan—the accurate peptide mass (m/z) were determined in the Orbitrap analyzer at 120,000 (FWHM, at m/z = 200) resolution. In the following MS2 scans (performed for peptide sequence identification), peptide ions were isolated with 0.7 m/z window, fragmented (CID, 35% NCE), and the fragment masses then determined in the Ion Trap analyzer (Turbo scan). In the following MS3 scans (performed for peptide quantification), peptide ions were isolated with 2 m/z window for MS2 fragmentation. Selected top 10 MS2 fragment ions (SPS) were further fragmented (HCD, 65% NCE), and the intensities of released reporter ions were determined in the Orbitrap analyzer at 50,000 (FWHM, at m/z = 200) resolution.

**Experimental Design and Statistical Rationale**

Experimental design (procedures in vivo and proteomics sample processing) is outlined in Fig. 1. Western blotting experiments were performed on two independent occasions. On each occasion, Western blotting data were obtained from cerebral artery samples of 2 control and 3 alcohol-exposed fetuses. ALDH activity assay was performed on 3 samples in each group: they were harvested from 2 control and 2 alcohol-exposed baboon fetuses. Considering the apparent effect of prenatal alcohol exposure on ALDH6A1 protein amount and function, the chosen number of observations within each group was enough to render statistically significant difference. Scarce availability of fetal baboon tissue precluded us from further exploration of the proteomics data set.

**Differential Protein Expression Analysis**

Reporter Ions Quantification approach based on TMT-labeling was employed for differential protein expression analysis as described below.

**Sample Processing**—Each sample containing 37 µg of extracted proteins in 75 µl of RIPA buffer was processed using a commercial kit (84840, Thermo Fisher) according to the manufacturer’s protocol with minor modification. The proteins were reduced with 10 mM DTT for 45 min at 50 °C, alkylated with 50 mM iodoacetamide for 20 min at RT in the dark, precipitated with 4 volumes of cold acetone (~20 °C, overnight), washed with 50 µl of cold (~20 °C) 90% acetone, air dried for 3 min and redissolved in 70 µl of digestion buffer (100 mM TEAB, pH 8.3). The dissolved proteins were digested with Lys-C enzyme (enzyme to protein ratio 1:100) for 2 h at 37 °C; the digestion was continued overnight in the presence of trypsin (enzyme to protein ratio 1:50). The peptide concentration was determined using a Pierce Quantitative Colorimetric Peptide Assay kit (Thermo Fisher) according to the manufacturer’s protocol.

**TMT Labeling**—Each reaction consisted of a set of 10 samples (Fig. 1B). Out of those, several samples represented “pools” of all available samples (control and experimental). Therefore, all pooled samples were identical and were used to assess technical replicability of the readings. Each sample containing 20 µg of peptides was labeled using commercial TMT-10plex Mass Tag Labeling reagent kit (90111, Thermo Fisher) according to the manufacturer’s protocol scaled to 50 µl sample volume. All samples within each reaction were labeled with different TMT tags to avoid bias in labeling and, therefore, potential bias in peptide/protein quantification. The labeled set of 10 samples was combined and vacuum dried; the combined 200 µg mixture of labeled peptides was reconstituted in 0.1% TFA at 0.3 µg/µl for further fractionation.

**Fractionation**—Three hundred microliters (90 µg) of labeled peptide mixture was fractionated using Pierce High pH Reversed-Phase Peptide Fractionation kit (Thermo Fisher) according to the manufacturer’s protocol—8 step fractions (consecutively eluted with 10.0, 12.5, 15.0, 17.5, 20.0, 22.5, 25.0, and 50.0% acetonitrile) were collected. The collected peptide fractions were vacuum dried.

**Post-acquisition Analysis of Raw MS Data—**Analysis was performed within a mass informatics platform Proteome Discoverer 2.2 (Thermo Fisher) using Sequest HT search algorithm and human and baboon protein databases (SwissProt, Homo sapiens, TaxID 9606, v.2017-05-10, 42,153 entries; SwissProt, Papio anubis, TaxID 9555, v.2017-05-10, 96 entries; TrEMBL, Papio anubis, TaxID 9555, v.2017-05-10, 21,637 entries). The reversed target databases were used as decoy databases. Full tryptic peptides were searched; 2 miscleavages were allowed. The search fixed modifications included: carbamidomethylation of Cys and TMT-6plex modification of Lys and any N terminus. The variable modifications included oxidation of Met and acetylation of the protein N terminus. The precursor, fragment, and reporter ion mass tolerances were set to 10 ppm, 0.6 Da, and 20 ppm.
respectively. The data were filtered for the precursor ions with S/N of
at least 1.5 and for peptide isotope pattern. The PSMs were filtered
using a delta Cn threshold of 0.05. The q-values were calculated at
PSM level (Percolator), and then at peptide level (Qvality algorithm)
to control false discovery rate (FDR). The FDR threshold of 0.01 was
used to validate and filter the identified peptide sequences. The
filtered/validated peptides were used for the identification of the
candidate precursor proteins. Each candidate precursor protein was
scored by summing the PEP values of the assigned peptides. The
sum-PEP protein scores were used to calculate the experimental
q-values at the protein level. The candidate proteins were validated
using 0.01 (strict) and 0.05 (relaxed) FDR thresholds. The following
criteria were applied to accept (or reject) the identification of a
candidate protein or a protein group. The identification of a can-
didate protein was accepted if at least one of the assigned peptides
was unique to that protein. A set of candidate proteins was ac-
ccepted as an identified protein group if none of the assigned
peptides was unique to any protein, but at least one of those
peptides was common and unique to that group of proteins. The
“best” protein of a group was displayed as Master protein; it
was selected for the highest parameter with the following priority: the
protein score, the number of assigned validated PSMs, the number of
assigned validated peptides, and its size (the number of residues).
The reporter ions quantification values were corrected based on the
product data sheet provided with the used TMT-10plex Label reagent
set; the co-isolation and average S/N ratio thresholds were set to
50% and 5, respectively. Total Peptide Amount option was selected
for the normalization of the samples. Unique peptides were used for
protein quantification; protein groups were considered for peptide
uniqueness. The abundances of identified proteins, the molar ratios
of identical proteins in compared samples, and corresponding statistics
were determined. The data were further analyzed at the UTHSC
Bioinformatics facility (see Data Analysis below).

SDS-PAGE and Western Blotting

Protein samples were loaded into 8–20% precast SDS-PAGE gel
(Bio-Rad), 30 μg total protein per well. After electrophoretic separa-
tion, proteins were transferred to 0.45 mm nitrocellulose membrane
(Bio-Rad). After transfer, membranes were incubated with 5% nonfat
milk as a blocking solution at room temperature for 1 h. For validation
of antibody specificity, the membrane was first incubated in blocking
solution containing a mixture of rabbit polyclonal anti-ALDH6A1
primary antibody (HPA029074, Sigma, 0.4 μg/ml) with 0.4 μg/ml of
corresponding immunogenic peptide (Appest78499, Sigma). The in-
cubation was performed overnight at 4 °C. For obtaining specific
binding, membranes were incubated in blocking solution containing
anti-ALDH6A1 primary antibody overnight at 4 °C. The next day, after
washing primary antibody with TBST, membranes were incubated with
goat anti-rabbit horseradish peroxidase (HRP)-conjugated sec-
secondary antibody at 1:5000 dilution in blocking solution. Incubation
was performed for 2 h at room T. Membranes were washed 3 times
with TBST for 10 min and probed with enhanced chemiluminescence
(ECL) reagent (Thermo Fisher) to obtain films using an X-ray developer
(SourceOne Healthcare technologies).

Aldehyde Dehydrogenase Activity Assay

Assay was performed using ALDH activity fluorimetric assay kit
(Cayman Chemical) following the manufacturer’s instructions.

Data Analysis

Differential Protein Expression Analysis—The data generated by
the mass spectrometry analysis was transferred to the UTHSC Mo-
olecular Bioinformatics Core using Secure File Transport Protocol.

Each multiplexed reaction (reaction 1 and reaction 2 representing a
combined set of ten individual samples labeled with TMT-10plex tags
for protein quantification) included replicates of control and experi-
mental samples and replicates of the same pooled sample. Two
multiplexed reactions were processed in parallel and analyzed for
protein identification and quantification. The abundances of all pro-
teins identified in a given individual sample were summed and nor-
malized relative to the sample with the maximum summed abundance
(i.e. the set of quantitative data collected for a given individual sample
was factorized so that the summed protein abundances were identical
in all the 20 individual samples). Within each multiplexed reaction, the
protein abundances determined in individual control and experimen-
tal samples were further normalized to the averaged abundance of
the replicate protein in the replicates of the pooled samples. Data
were normalized using normalized cyclic loss function from R/Bio-
conductor-package limma (51). The normalized data matrix was
loaded into R to gather statistics and determine differential expres-
sion. Mean, variance, standard deviation, and standard error of the
mean were calculated for each protein across each condition. Pear-
son’s correlation coefficient was graphed in order to identify sample
outliers in each condition. At the time of analysis no clear outliers were
identified. A principle component analysis was performed to identify
different clusters in the samples.

Having determined the mean abundances of the proteins identi-
ified/quantified in replicates of the control and the experimental sam-
ple, the protein fold changes (experimental over control) were
calculated. The described processing of quantitative data was per-
formed in Log2 format. The means of analytical conditions (experi-
mental, control) were subtracted to obtain the Log2 fold change. This
calculation was then transformed to linear space to obtain the fold
change. Welsh’s t test was implemented in order to determine sig-
nificance for each protein. Then, p values were adjusted for multiplic-
ity using the Benjamini-Hochberg method (52). Only proteins with an
adjusted p value < 0.05 were considered differentially expressed. These
proteins were used to create a heatmap with unsupervised

Protein Analysis Using PANTHER—Analysis was performed using
PANTHER13.1 software (pantherdb.org). Protein class analysis was
performed using built-in functional classification in PANTHER. The
Homo sapiens gene database was used as a reference. Annotations
were performed according to PANTHER Protein Class.

Protein-Protein Interaction Network and Statistical Enrichment
Analysis—The list of proteins with statistically significant differences
in abundance between samples from control versus alcohol-exposed
fetuses was analyzed using Fisher’s exact test with FDR multiple test
correction in PANTHER13.1 software (pantherdb.org). Enrichment
was accepted with the false discovery rate (FDR)<0.05.

Western Blots—Intensity of the bands was quantified using ImageJ
1.46r software (http://imagej.nih.gov/ij/). Optical densities of the
ALDH6A1-corresponding bands from control versus alcohol-treated
groups were normalized to corresponding anti-KCNMB1 bands and
normalized values were compared using Mann-Whitney test with
two-tail p value in InStat3 (GraphPad). Statistical significance was set
at p < 0.05.

Final Plotting—Graphs were prepared using Origin 8.5.1 (Origin-
Lab; Northampton, MA).

RESULTS

Protein Detection and Identification—Three controls and
four alcohol-exposed fetuses were used as artery donors (Fig.
1A). Cerebral (basilar) arteries and their first order branches
from near-term baboon fetuses were visually identified as
surface vessels on the inferior side of the fetal cerebellum.
Under a microscope, up to 1 g of arteries was dissected out under a microscope from each donor and gently rinsed with phosphate-buffered saline to minimize contamination with blood components. After rinsing, arteries were cleaned of extra-arterial tissue under the microscope. From each fetus, arteries were split into two samples, which were treated as replicates. Artery lysates were prepared in a reproducible manner as described in Materials and Methods. The identity of artery lysates was validated in the few randomly picked samples by Western blot immunostaining against the endothelial cell marker platelet endothelial cell adhesion molecule-1 (CD31), compared with lack of this staining in lysates of fetal cerebellum nervous tissue (supplemental Fig. S1).

Following protein expression detection procedures, a total of 6161 protein groups were identified, with 5943 protein groups being quantified. Out of those, 5424 proteins were identified with high confidence (FDR < 0.01), 397 with medium confidence (FDR < 0.05), and 122 with low confidence (FDR > 0.05) (supplemental Table S1). All quantified proteins were analyzed using PANTHER software to determine protein distribution by class per standard PANTHER class definitions. PANTHER analysis yielded a total of 846 gene hits and 906 class hits corresponding to our protein list. By class, the most abundant proteins represented nucleic acid binding (178 genes), followed by classes of enzyme modulators (99 genes) and hydrolases (99 genes) (Fig. 2A).

Proteome Differential Analysis—Out of 5943 proteins, 4953 were quantified in all samples, and the abundance of these proteins was subjected to further statistical analysis, i.e. differential analysis at UTHSC Bioinformatics facility (see Materials and Methods). Before differential analysis, the abundance measurements of the identified proteins with quantified abundance were used for a principle component analysis (PCA). No apparent clustering of samples was observed (supplemental Fig. S2A–S2B). The normalized data matrix was loaded into R software environment to determine differential expression of the proteins when comparing control versus experimental samples. A total of 238 proteins were detected with abundances that differed significantly between control and experimental samples. Out of these 238 proteins, none belonged to the group of proteins that were identified with medium confidence.

The abundance measurements of the differentially expressed proteins were used to calculate Pearson’s correlation coefficients (supplemental Fig. S2C). The control and experimental samples did cluster only when using differentially expressed proteins (supplemental Fig. S2D). PCA analyses revealed that experimental sample two (E2) clustered in a similar pattern to the controls (supplemental Fig. S2D). Protein abundance heatmap further confirmed preferential clustering of experimental sample 2 (E2) with controls as opposed to experimental samples. However, when compared within the control group, replicates from E2 clustered separately from controls (supplemental Fig. S3). Overall, protein abundance heatmaps revealed close clustering or replicates from each animal donor. This outcome demonstrated the low technical variability of our approach.

Fig. 1. Experimental design. A, Alcohol gastric infusion procedure in vivo. Pregnant dams were subjected to gastric infusion of either control or alcohol-containing drink three times during second trimester-equivalent (90 ± 5, 100 ± 5, and 110 ± 5 dGa out of 175 days of term pregnancy in baboons). Fetal cerebral (basilar) arteries were harvested following cesarean section near-term (165 ± 5 dGa). Cerebral artery tissue from each fetal baboon donor was divided by half to be processed for proteomics analysis as a duplicate. BAC: blood alcohol concentration; dGa: days of gestational age. B, Samples for proteomics analysis were run in duplicates on two different plates (reaction 1 and reaction 2). The only exception was sample from one alcohol-exposed fetus where duplicates had to be combined to render desired protein amount (E3.1 + E3.2). Three wells in one plate and four wells in another plate were used for reading of identical pooled samples (P1.1, P2.1, P3.1, P4.2, P5.2, P6.2, and P7.2) to assess the intra-plate (within reaction) variability of the readings. Contents of each well were differentially tagged for LC-MS using TMT-10plex Mass Tag labeling kit (Material and Methods). The variance in protein readings from the pooled samples was found to be minimal. The variance between the pooled samples in reaction 1 was ranging from 2.51e-07 to 0.41 (standard deviation ranging from 5.02e-04 to 0.64). The range of variance between the pooled samples in reaction 2 was 4.68e-06 to 0.377 (standard deviation ranging from 2.16e-3 to 0.61).
Class analysis by PANTHER applied to 238 proteins with differential expression between control and experimental samples showed that the most abundant proteins corresponded to transferases (22 genes) and nucleic acid binding proteins (20 genes), which were followed by enzyme modulators (17 genes), cytoskeletal proteins (16 genes), hydrolases (16 genes), and oxidoreductases (15 genes) (Fig. 2B).

It is noteworthy that the class distribution of differentially expressed proteins did not represent a smaller replica of the class distribution of all identified proteins (Fig. 2A versus 2B). These data suggest that fetal cerebral artery proteins were affected by prenatal alcohol exposure with some selectivity.

Protein Representation Within the Groups of Significantly Upregulated Versus Downregulated Proteins—All 238 proteins that were differentially expressed between control and experimental groups (supplemental Table S2) were divided into two groups: proteins that were significantly upregulated (132 proteins, supplemental Table S2, red) and downregulated (106 proteins, supplemental Table S2, blue) by prenatal alcohol exposure. Upregulated proteins were mostly transferases (19 genes) and oxidoreductases (13 genes) (Fig. 3A). These two classes accounted for nearly 25% of all upregulated proteins. Remarkably, the biggest class within the downregulated proteins was represented by cytoskeletal proteins (14 genes or 13% of all downregulated proteins) (Fig. 3B). In the group of downregulated proteins, transferases (3 genes) and oxidoreductases (2 genes) accounted for less than 5% of all downregulated proteins. These data further support the notion regarding selective targeting of proteome members by prenatal alcohol exposure.

Protein Representation Within the Group of Differentially Expressed Proteins—Analysis of protein-protein interactions in STRING10.5 (string-db.org) yielded \( p < 1.0e^{-16} \). This value pointed at the fact that the list of differentially expressed proteins had more interactions that would be expected from a
randomly picked protein sample. Rather, differentially expressed proteins represented the proteins that were connected into a biological group. Indeed, network analysis revealed that differentially expressed proteins contained several large networks (Fig. 4). The largest one contained 58 genes and was representative of cellular processes and cytoskeleton (Fig. 4, area shaded in soft pink). It was closely followed by another network (38 genes) that was presented by catalytic activity and metabolic processes. Smaller networks (8–16 genes) were presented by integrin signaling pathway (extracellular/matrix location) and ubiquitin proteasome pathway. Overall, protein-protein interaction networks in STRING software and data obtained during protein analysis by biological function, location, and molecular process in PANTHER yielded similar results. Both approaches identified cellular metabolism and structure as major targets of alcohol in fetal cerebral arteries.

Next, we applied enrichment analysis that was directed toward characterization of protein biological function, location, and role in molecular processes for 238 proteins that were differentially expressed between control and experimental samples.
Enrichment analysis revealed that differentially expressed proteins were vastly involved in metabolic processes (Fig. 5). Further, the differentially expressed proteins were presented by extracellular matrix (laminin complex), but also resided within cells and cellular organelles, such as mitochondria (Fig. 6). Differentially expressed proteins possessed structural, binding and enzymatic activities (Fig. 7A). Finally, enrichment analysis pointed at metabolic pathways as the major constituent within the group of differentially expressed proteins (Fig. 7B).

Expression and Functional Verification of Proteomics Data—For validation of proteomics analysis, we chose aldehyde dehydrogenase 6 family member A1 protein. This protein is in mitochondria and catalyzes the irreversible oxidative decarboxylation of malonate and methylmalonate semialdehydes to acetyl- and propionyl-CoA (53). Western blotting revealed that the optical density of ALDH-associated band normalized to KCNM1-associated signal in basilar artery lysates from alcohol-exposed fetuses was 1.25 times higher than in samples from control fetuses ($p = 0.01$) (Fig. 8A–8B).

Finally, we wanted to determine whether upregulation of the protein amount translated into increased total function of ALDH in arteries from alcohol-exposed fetuses. ALDH activity fluorimetric assay failed to detect measurable ALDH activity in basilar artery lysates of control fetuses. The same assay detected activity of the ALDH enzyme in basilar artery lysates of alcohol-exposed fetuses (Fig. 8C). The assay exhibited a high degree of reproducibility, as two out of three control samples were collected from the same fetal baboon donor,
and both were unable to yield measurable ALDH activity. In contrast, two out of three experimental samples were collected from the same alcohol-exposed fetal baboon donor, and both showed detectable ALDH activity of 0.15 and 0.16 nM/min/ml.

DISCUSSION

To our knowledge, the current work constitutes the first documentation of proteome changes in fetal cerebral arteries following prenatal alcohol exposure. We demonstrated that alcohol exposure during mid-pregnancy led to significant changes in the expression of 238 proteins. These proteins largely represent cellular proteins enabling metabolic processes. Remarkably, these changes were observed following only three episodes of binge alcohol exposure. During these episodes, maternal blood alcohol concentration averaged 80 mg/dL (17 mM). This concentration represents the legal limit of alcohol intoxication for driving a motor vehicle in most of the United States. Considering that lethal blood alcohol levels in humans average 355 mg/dL (54), BAC in our study constitutes a rather modest, yet clinically relevant amount. We used the baboon fetal basilar artery, which provides blood supply to the cerebellum, brainstem, and occipital lobes. In general, baboon fetal development and cerebral circulation are strikingly like humans (55). With regards to our choice of blood vessel, the basilar artery represents a rather sensitive target for prenatal alcohol exposure: in ovine model of pregnancy, fetal alcohol intoxication resulted in selective increase of cerebral blood flow in cerebellum, with subsequently detected neuronal loss in this region (7).

In our work, changes in cerebral artery proteome were detected in near-term baboon fetuses (165 dGa), that is, 55 days after the last alcohol exposure episode took place (Fig. 1). These findings support the interpretation that effects produced by prenatal alcohol exposure on fetal cerebrovascular function could have a long-lasting nature. This agrees with a recent report in mice: animals that were prenatally exposed to alcohol exhibited significantly greater motor deficit when compared with the control group, during recovery from middle cerebral artery occlusion performed three months after the last alcohol exposure (8).

One of the most novel and striking findings of the current work is the identification of the mitochondria-associated proteins as targets for prenatal alcohol exposure within the fetal cerebral artery. Mitochondrial electron transport constitutes the major intracellular source of reactive oxygen species (ROS), and ethanol treatment imposes conditions that promote ROS formation by mitochondria in the liver and de-
In cerebellar neurons, mitochondria have been implicated in alcohol withdrawal symptoms. It has been proposed that the neuronal hyperexcitability that is observed during alcohol withdrawal promotes an increase in intracellular calcium levels and a decrease in a calcium binding protein, both of which result in excessive entry of calcium into the mitochondria. The resulting increase in ROS, in turn, would impede ATP generation (57). In cardiomyocytes, alcohol produced concentration- and time-dependent increases in mitochondrial ATP-synthase without alteration of the mRNA profile (58). In cerebral arteries, however, the effect of alcohol on mitochondrial function is unknown. Moreover, mitochondrial function has only recently emerged as a critical regulator of cerebral artery physiology and pathology (59). In our study, modifications in fetal mitochondrion proteome are found to be sensitive to relatively brief and/or infrequent exposure to alcohol. Moreover, these modifications are observed weeks after alcohol is removed from fetal and maternal circulation. Indeed, although fetal alcohol exposure in our experimental protocol occurred during the second trimester equivalent of pregnancy (90–110 dGa in baboons), proteomic analysis detected changes in mitochondrial proteome near term (165 dGa). There are two possibilities that may underlie this phenomenon. First, the mitochondrial proteome is modified by alcohol immediately, but these changes are long-lasting and can be observed near term. Sensitivity of mitochondrial function to acute alcohol exposure observed in various cell types and organs certainly supports this possibility (56, 58). Second, the changes in mitochondrial proteome represent an adaptive response to alcohol exposure, this adaptive change requires time to develop. These two possibilities require experimental interrogation. Moreover, consequences of alcohol-driven alterations in mitochondrial proteome on cerebral artery function in utero and in postpartum development remain to be established.

Prenatal alcohol exposure (PAE) of Sprague-Dawley rats to a 3% ethanol liquid diet for the duration of the pregnancy led to attenuation of cerebral arterial responses to endothelial and neuronal nitric oxide synthase-dependent pharmacological modulators at 4–6 weeks after birth (35). The response was recovered by the antioxidant apocynin, suggesting the critical role of ROS in the vascular consequences of prenatal alcohol exposure (35). Considering that, in adult cerebral arteries, mitochondrial function con-
In the current work, we detected upregulation of ALDH enzymatic activity in basilar artery lysates from alcohol-exposed fetuses (Fig. 8C). This increase in enzymatic activity is consistent with the upregulation in ALDH6A1 protein expression, as detected by proteomics analyses (supplemental Table S2) and further validated by Western blotting (Fig. 8A–8B). Deficiency in ALDH6A1 activity has been implicated in dysmyelination and transient methylmalonic aciduria (60). On the other hand, elevated ALDH activity is required for maintenance of drug-resistant tumors (61). Conceivably, down-regulation of the ALDH6A1 gene correlated with progression-free status of colon cancer patients in a clinical setting (62). The link between ALDH6A1 activity and cancer is of interest, as prenatal alcohol exposure has been recently implicated in increased susceptibility to carcinogenesis and tumor progression (63).

Unlike mitochondrial enzymes, cellular structural components presented downregulated protein group in arteries from alcohol-exposed fetuses (Fig. 3B). This outcome may explain previous observations showing decreases in microvessel density in a mouse model following daily subcutaneous injections of pregnant dams with 0.5, 1, or 3 g/kg ethanol administered from gestational days 13 to 19 (64). This timeline coincides with our experimental paradigm (i.e. prenatal alcohol exposure during second trimester equivalent of human pregnancy), as a 13 to 19 dGa period in a mouse model covers second trimester equivalent in humans (43). Loss of microvessel density is accomplished by the disruption of cell adhesion and cytoskeleton protein networks. Unlike microvessels, cerebral arteries do not seem to exhibit apparent morphological deficiency, yet their cytoskeleton and extracellular matrix still undergo major changes (Fig. 6). The cellular location of changes in structural/extracellular matrix proteins identified in our work remains unknown and may be presented by all cell types (such as endothelium, vascular smooth muscle, fibroblasts) described for cerebral arteries (65).

In the current work, we used a gastric route of alcohol administration. Gastric administration is advantageous compared with intravenous infusions because the former replicates the route of alcohol consumption by humans. However, alcohol gastric administration does not allow answering the question whether ethanol itself or rather its metabolites serve as molecular triggers of proteome changes observed in our analysis. For example, impairment of cerebral microvesSEL endothelium autophagy by PAE in mouse fetuses was ablated by 4-methylpyrazole, which blocked formation of the ethanol metabolite acetaldehyde (66). The role of ethanol metabolites in the ultimate effects of PAE on the fetal cerebral artery proteome remains to be established.

Despite the power of proteomics analysis and use of a nonhuman primate model of alcohol consumption during pregnancy, there are potential limitations to our work. First, in our experimental paradigm, alcohol was administered under maternal anesthesia (induction with ketamine, maintenance with isoflurane; see Methods). Although the control group received control drink under anesthesia as well, we cannot rule out that some of the observed effects originate from interaction of anesthetic agents and alcohol targets. Interactions of alcohol with various anesthetics are noted in the literature (67–69). However, the use of anesthesia was essential for the following reasons. 1) The use of anesthesia allowed functional studies of fetal cerebral artery using Doppler sonography of baboon dams (10–11). 2) We were able to standardize the timing and amount of alcohol delivery, and both would be difficult to achieve in voluntary drinking animals, because the development of alcohol preference takes over a month in an anesthesia-free, “food-induced” drinking paradigm in adult baboons (70). In addition, such a paradigm renders highly variable BACs (70), and thus would require a much larger number of animals to achieve reliable results. 3) Regarding direct applicability of our experimental paradigm to humans, it is estimated that 1–2% of pregnant women undergo surgical anesthesia during pregnancy (71). Some of these cases may include emergency intervention while the prospective mother is under alcohol intoxication. It is noteworthy that our findings identifying mitochondrial and structural proteins as major targets of PAE may adequately explain previous observations that were obtained in anesthesia-free animal models (35). Formal testing of the direct applicability of our findings to a human population is being planned. Such testing may obviate the limitation of alcohol administration under maternal anesthesia and possible (yet unlikely) species differences in alcohol effects in Papio versus Homo.

Although nonhuman primates (including baboons) are widely used in various studies of pathophysiology and alcohol consumption (70, 72), one of the major limiting factors of primate use is the scarce availability of experimental animals. The scarce availability of fetal baboon tissue precluded us from performing large-scale validation of proteomic results with Western blotting and functional assay. Validation of proteomics data with other techniques is critical, as proteomics analysis is notorious for rendering false-positive observations (73), and as such constitutes a second potential limitation of our study. Moreover, some of the differentially expressed proteins showed very modest fold-change (1.1–1.2) (supplemental Table S2). We have tried to overcome this limitation by using data normalization. In addition, randomly picked ALDH6A1 protein for validation revealed upregulation of ALDH6A1 amount and ALDH function (Fig. 8) as expected from proteomics data (supplemental Table S2). Finally, the enrichment analysis pointed at the statistically significant difference in the number of protein interactions within our protein group when compared with a set of randomly sampled
proteins (see Results above). Thus, it is highly unlikely that our data represented a false or random observation.

On a final note, two large protein groups (metabolism and structure) identified in cerebral arteries as targets of PAE, are strikingly like data from a global chemo-biology approach used to analyze murine neuronal tissue following PAE (74). This similarity raises the question as to whether PAE exerts universal effect on various tissues/organisms.

In summary, using a nonhuman primate model of pregnancy, we established significant proteome changes within fetal cerebral artery in response to PAE for the first time. These changes were observed long after the last alcohol exposure episode and were mostly represented by proteins involved into the cellular metabolism.

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DATA AVAILABILITY

The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomexchange.org) via the Massive partner repository (ftp://massive.ucsd.edu/MSV000082291; Center for Computational Mass Spectrometry, Computer Science and Engineering, University of California at San Diego) with the data set identifier PXD009545.

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