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IMPAIRED PLACENTATION IN FETAL ALCOHOL SYNDROME

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Abstract

Introduction—Intrauterine growth restriction (IUGR) is one of the key features of fetal alcohol syndrome (FAS), and IUGR can be mediated by impaired placentation. Insulin-like growth factors (IGF) regulate placentation due to stimulatory effects on extravillous trophoblasts, which are highly motile and invasive. Previous studies demonstrated that extravillous trophoblasts express high levels of aspartyl-(asparaginyl) β -hydroxylase (AAH), a gene that is regulated by IGF and has a critical role in cell motility and invasion. The present study examines the hypothesis that ethanol impaired placentation is associated with inhibition of AAH expression in trophoblasts.

Methods—Pregnant Long Evans rats were fed isocaloric liquid diets containing 0% or 37% ethanol by caloric content. Placentas harvested on gestation day 16 were used for histopathological, mRNA, and protein studies to examine AAH expression in relation to the integrity of placentation and ethanol exposure.

Results—Chronic ethanol feeding prevented or impaired the physiological conversion of uterine vessels required for expansion of maternal circulation into placenta, a crucial process for adequate placentation. Real-time quantitative RT-PCR analysis demonstrated significant reductions in IGF-I, IRS-1, IRS-2, and IRS-4, and significant increases in IGF-II and IGF-II receptor mRNA levels in ethanol-exposed placentas. These abnormalities were associated with significantly reduced levels of AAH expression in trophoblastic cells, particularly within the mesometrial triangle (deep placental bed) as demonstrated by real time quantitative RT-PCR, Western blot analysis, ELISA, and immunohistochemical staining.

Conclusions—Ethanol-impaired placentation is associated with inhibition of AAH expression in trophoblasts. This effect of chronic gestational exposure to ethanol may contribute to IUGR in FAS.

Keywords

Fetal alcohol syndrome; placenta; insulin-like growth factor; placentation; aspartyl-(asparaginyl) β -hydroxylase

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INTRODUCTION

Maternal consumption of alcohol during pregnancy is one of the most common and preventable causes of birth defects and developmental disabilities in United States. Fetal Alcohol Spectrum Disorders (FASD) is the umbrella term for the range of structural malformations and behavioral and neuro-cognitive disabilities produced by different degrees and timing of prenatal ethanol exposure [1]. Recent studies linked teratogenic central nervous system (CNS) effects of chronic ethanol exposure to impairments in insulin and insulin-like growth factor (IGF) signaling mechanisms that mediate neuronal growth, survival and migration during development [2,3]. In addition to CNS abnormalities, chronic gestational exposure to ethanol causes intrauterine growth restriction (IUGR) [4–7], the pathogenesis of which is unknown. However, since IGFs regulate placentation due to their stimulatory effects on extravillous trophoblasts [8–10], which are highly motile and invasive, ethanol inhibition of IGF signaling mechanisms may contribute to IUGR by impairing placentation.

Previous studies demonstrated that extravillous trophoblasts express high levels of aspartyl- (asparaginyl) β -hydroxylase (AAH), a gene that is regulated by IGF and has a critical role in cell motility and invasion. AAH is an α -ketoglutarate-dependent dioxygenase that catalyzes post-translational hydroxylation of β carbons of aspartyl and asparaginyl residues present in epidermal growth factor-like domains of proteins such as Notch and Jagged, which have demonstrated roles in cell migration [11–14]. AAH is abundantly expressed in many malignant neoplasms [15,16], and high levels of AAH correlated with increased motility and invasive growth [17]. In this regard, over-expression of AAH in transfected cells results in increased motility and invasiveness, while antisense or siRNA silencing of AAH inhibits cell motility [17–20]. AAH expression is regulated by insulin and IGF signaling downstream through insulin receptor substrate (IRS) proteins and PI3 kinase-Akt or Erk MAPK [17,20].

Placenta is the only non-transformed tissue that exhibits very high levels of AAH expression [15]. Recent studies localized AAH immunoreactivity to trophoblasts, with the most abundant levels detected in extravillous trophoblasts, which mediate placentation by way of their highly motile and invasive properties [21]. Since IGFs stimulate motility and invasiveness of trophoblasts, and ethanol inhibits IGF signaling, we hypothesize that ethanol induced IUGR is mediated by impaired placentation and associated with reduced expression of downstream targets of IGF stimulation, e.g. AAH. In the present study, using a chronic gestational ethanol exposure model, we examined the effect of ethanol on placental morphology, including deep placental bed, expression of genes in the IGF signaling cascade, and the levels of AAH expression/immunoreactivity in trophoblasts.

METHODS

In vivo model of chronic ethanol exposure: Pregnant Long Evans rats were fed with isocaloric liquid diets (BioServ, Frenchtown, NJ) in which ethanol comprised 0% (N=6) or 37% (N=10) of the caloric content [22]. An additional control group (N=2) in which chow is supplied ad libitum was included to control for changes associated with the liquid diet. The liquid diets were initiated on gestation day (GD) 6, which was calculated from the appearance of vaginal copulatory plug, and continued throughout pregnancy. This level of ethanol consumption produces blood concentrations between 50 and 60 mM [3], comparable to the serum levels of chronic alcoholics [23]. Rats were monitored daily to ensure equivalent caloric consumption and maintenance of body weight. The implantation sites were dissected and the placental tissue with underlying mesometrial triangle was harvested on GD 16. Placenta and pup weights were documented in all groups. The experiments were repeated 3 times. Since the response to ethanol in pups/placentas generated from the same litter was heterogeneous, while the response between different litters was homogenous, we designated “N” as the number of placentas

analyzed instead of number of dams. Fresh placental tissue with attached mesometrial triangle was snap frozen in a dry ice-methanol bath and then stored at -80°C for mRNA and protein studies. In addition, placentas with underlying mesometrial triangle were immersion fixed in Histochoice fixative (Amresco Corp., Solon, OH) and embedded in paraffin. In order to compare the AAH expression in placenta to mesometrial triangle, in 2 dams per group, we separated the placenta from the implantation site at the time of tissue harvest. After stripping of the decidua basalis from the placental disc, the placenta was snap frozen. Then we carefully dissected the mesometrial triangle from the uterus, and since the extravillous trophoblastic cells invade through both the decidua basalis and mesometrial triangle, we combined these tissues per sample and snap frozen together. Snap frozen placental tissue and mesometrial triangle tissue with the decidua basalis were utilized for comparative analysis of AAH mRNA and protein expression studies. Isolation of the structures was verified by histological examination of a portion of each specimen.

Morphometric analysis

Paraffin embedded histological sections ($5\mu\text{M}$ thick) through the complete implantation site were stained with hematoxylin and eosin and examined by light microscopy. Two placentas per litter from 3 control ($N=6$) and 3 ethanol-exposed ($N=6$) dams were included in the evaluation. The morphometric analysis of the placentas with underlying mesometrial triangles was performed using ImagePro Plus software (Media Cybernetics, Inc., Silver Spring, MD). Digital images were acquired from control and ethanol-exposed placentas with mesometrial triangles. Using reference calibrations for total magnification of our optical system (Olympus BX60; Olympus America Inc., Center Valley, PA), we measured the maximum thickness of the labyrinthine plus junctional zone as thickness of placentas. The maximum perpendicular line from the apex of the mesometrial triangle to the junctional zone was measured as the thickness of the mesometrial triangle in each placenta, which included decidua basalis. In order to evaluate the effects of gestational ethanol exposure on vascular transformation, we randomly selected 5 cross sections of maternal spiral artery in the mesometrial triangles of 6 control and 6 ethanol-exposed placentas. Using image analysis and reference calibration as described above, the circumference of the vessels was measured and the mean circumference was calculated for each placenta. The cellularity of mesometrial triangle was quantified from 3 random high magnification foci (400X) of each placenta by counting the nuclei. Number of trophoblast giant cells was quantified in a similar fashion from 5 random low magnification foci (100X) of each placenta.

Immunohistochemical staining

Paraffin embedded placental tissue sections ($5\mu\text{m}$ thick), which included underlying mesometrial triangle, from 4 control and 4 ethanol exposed dams (3 per litter; $N=12$) were immunostained to detect AAH immunoreactivity. Prior to immunostaining, the sections were deparaffinized in xylenes, rehydrated in graded alcohol solutions, and treated with 0.1 mg/ml saponin in phosphate buffered saline (10 mM sodium phosphate, 0.9% NaCl, pH 7.4; PBS) for 20 minutes. To quench the endogenous peroxidase activity, the sections were treated with 0.6% hydrogen peroxide in methanol at room temperature for 20 minutes. The tissue sections were then incubated with Avidin-Biotin blocking reagents (Vector Laboratories, Burlingame, CA) for 15 minutes, followed by 0.5% normal horse serum prepared in SuperBlock-TBS (Pierce Biotechnology Inc., Rockford, IL) for 30 minutes to block non-specific binding sites. After overnight incubation at 4°C with $1\mu\text{g/ml}$ of FB50 monoclonal AAH antibody [15,24], immunoreactivity was detected using biotinylated secondary antibody, avidin biotin horseradish peroxidase complex (ABC) reagents, and diaminobenzidine as the chromogen (Vector Laboratories, Burlingame, CA). The sections were lightly counterstained with hematoxylin and examined by light microscopy.

Western blot analysis

Western blot analysis was used to examine AAH protein expression in placental tissue. Fresh, snap frozen samples of 4 placentas with underlying mesometrial triangles per litter from 2 control and 2 ethanol exposed dams (N=8) were homogenized in 5 volumes of radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA) containing protease (1 mM PMSF, 0.1 mM TPCK, 1 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 1 mM NaF, 1 mM Na₄P₂O₇) and phosphatase (2 mM Na₃VO₄) inhibitors. The supernatant fractions obtained after centrifuging the samples at 12,000 × g for 15 minutes at 4°C were used for Western blotting. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Protein samples (40 µg) were fractionated by sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) [16]. After transferring the proteins to Polyvinylidene Difluoride (PVDF) membranes using a semidry transfer apparatus, non-specific binding sites were adsorbed with SuperBlock-TBS (Pierce, Rockford, IL). The membranes were incubated overnight at 4°C with the primary A85G6 monoclonal antibody to AAH (0.5–1 µg/ml) diluted in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH: 7.4) containing 1% bovine serum albumin, 0.05% Tween-20 (TBST-BSA), and 0.025% NaN₃. After thorough rinsing in TBST, the blots were incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:30,000) diluted in TBST+0.5% Casein. Immunoreactivity was revealed using SuperSignal enhanced chemiluminescence reagents (ECL; Pierce Chemical Company, Rockford, IL) and quantified by digital imaging with the Kodak Digital Science Imaging Station (NEN Life Sciences, Boston, MA). To demonstrate equal loading of samples, the blots were stripped and re-probed with polyclonal antibody to p85 protein (subunit of PI3 kinase).

AAH Enzyme-linked immunosorbant assay (ELISA)

An ELISA was constructed to compare the AAH immunoreactivity in placental and mesometrial triangle tissue homogenates. Assays were conducted in replicates of 3 and using 20 placentas and 20 corresponding mesometrial triangles with deciduas basalis selected from 2 dams per group. The separated mesometrial triangle including decidua basalis and placental tissue samples homogenized in RIPA buffer as described above were adsorbed (50 ng in 100 µl TBS) to the flat surfaces of opaque white polystyrene 96-well plates (Packard Optiplat-96, Perkin Elmer, Boston, MA) overnight at 4°C. Between steps, the wells were washed 3 times with TBST using a Nunc Immunowash apparatus (Nunc, Rochester, NY). Non-specific binding sites were blocked by filling the wells with 300 µl 1% BSA in TBST, and incubating at room temperature for 3 hours with gentle platform agitation. AAH proteins were detected by incubating the samples for 1 hr at 37°C with 0.5 µg/ml of A85E 6 monoclonal antibody generated to recombinant AAH protein. As a negative control, parallel reactions were incubated with non-relevant monoclonal antibody to Hepatitis B virus surface antigen (HBVSAg). Antibody binding was detected by incubating the reactions for 1 hr at room temperature with 100 µl/well of HRP-conjugated anti-mouse IgG diluted 1:10,000 in TBST. Subsequently, the reactions were incubated with SuperSignal Pico West Chemiluminescence Substrate (Pierce Biotechnology, Rockford, IL), and luminescence (relative light units; RLU) was measured in a TopCount machine (Packard Instrument Company, Meriden, CT). Specific binding was calculated by subtracting non-specific binding (HBSAg RLU values) from corresponding AAH binding (A85E6 RLU values).

Real time quantitative RT-PCR assays

Real time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to demonstrate AAH, IGF-I, IGF-II, insulin, IGF-I, and IGF-II receptors, insulin receptor substrate (IRS) types 1, 2, and 4 mRNA expression levels in control and ethanol exposed

placental tissue with underlying mesometrial tissue (4 samples per litter; 2 dams per group; N=8). In addition, to compare the AAH expression in placenta to mesometrial triangle, we utilized the tissue that was separated into placenta and mesometrial triangle with decidua basalis at the time of tissue harvest (5 samples per litter; 2 dams per group; N=10). Total RNA was isolated from the snap frozen placental tissue using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA concentration and purity were determined from the absorbances measured at 260 nm and 280 nm. Samples containing 2 µg RNA were reverse transcribed with the AMV First Strand cDNA synthesis kit (Roche, Basel, Switzerland) and random oligodeoxynucleotide primers. PCR primers for insulin (IN), IGF-I, and IGF-II growth factors and their corresponding receptors; insulin receptor substrates (IRS)-1, IRS-2, IRS-4; and AAH were designed using Mac Vector 7.0 software (Accelrys Inc., Oxford Molecular Ltd., Oxford, England). PCR amplifications were performed in 25 µl reactions containing cDNA generated from 2.5 ng of original RNA template, 300 nM each of gene specific forward and reverse primer (Table 1), and 12.5 µl of 2x QuantiTect SYBR Green PCR Mix (Qiagen Inc, Valencia, CA). The amplified signals were detected continuously with the BIO-RAD iCycler iQ Multi-Color RealTime PCR Detection System (Bio-Rad, Hercules, CA). The amplification protocol used was as follows: initial 15-minutes denaturation and enzyme activation at 95°C, 45 cycles of 95°C × 15 sec, 60°C × 30 sec, and 72°C × 30 sec. Annealing temperatures were optimized using the temperature gradient program provided with the iCycler software. Experiments were performed in triplicate.

Primer-target specificity was checked using NCBI-BLASTn (Basic Local Alignment Search Tool–nucleotide) searchHES. In addition, SYBR Green-labeled PCR products were evaluated by agarose gel electrophoresis, and the authenticity of each amplicon was verified by nucleic acid sequencing. The complementary (c) DNAs were cloned into the PCRII vector (Invitrogen, Carlsbad, CA). Serial dilutions of known quantities of recombinant plasmid DNA containing the specific target sequences were used as standards in the PCR reactions, and the regression lines generated from the C_t values of the standards were used to calculate mRNA abundance. Ribosomal 18S RNA levels measured in parallel reactions were used to calculate relative abundance of each mRNA transcript [22,25]. Results were normalized to 18S because 18S is highly abundant and the levels were essentially invariant among samples, whereas housekeeping genes were modulated by disease state. Control studies included real-time quantitative PCR analysis of: 1) template-free reactions; 2) RNA that had not been reverse transcribed; 3) RNA samples that were pre-treated with DNase I; 4) samples treated with RNase A prior to reverse transcriptase reaction; and 5) genomic DNA.

Statistical analysis

Data are depicted as means ± S.E.M. in the graphs. Intergroup comparisons were made using Student T tests or chi-square or analysis of variance (ANOVA) with the Tukey-Kramer post-hoc test for significance. Statistical analyses were performed using the Number Cruncher Statistical System (Kaysville, UT). Significant P-values (< 0.05) are indicated over the graphs.

Source of reagents

The FB50 monoclonal antibody was generated by immunizing mice with FOCUS hepatocellular carcinoma cell line and was used to isolate human AAH from a cDNA expression library [26,27]. The FB50 antibody binds to the N-terminus of AAH protein. The A85G6 and A85E6 monoclonal antibodies were made with recombinant Baculovirus generated protein and binds to the catalytic domain of AAH protein. All the AAH antibodies were generated by Liver Research Center, Rhode Island Hospital, Providence, RI. QuantiTect SYBR Green PCR Mix was obtained from Qiagen Inc. (Valencia, CA). All other fine chemical and reagents were purchased from Calbiochem-EMD Biosciences (La Jolla, CA), Pierce Biotechnology Inc. (Rockford, IL), or Sigma-Aldrich (St. Louis, MO).

RESULTS

Chronic gestational exposure to ethanol increases fetal resorption. We used a well-established experimental FAS model in which pregnant Long-Evans rats were fed with isocaloric liquid diets where ethanol comprised 0% or 37% of the caloric content [22]. Diets were initiated on gestation day (GD) 6, which was calculated from the appearance of the vaginal copulatory plug, and continued throughout the gestation. An additional control group (N=2) in which chow is supplied ad libitum was included to control for changes associated with the liquid diet. The placentas were harvested on GD 16. The litter size was 13 and 16 in the chow group (N=2). The litter size in control group (N=6) ranged from 6-to-13. We detected pregnancy loss in 4 of 10 (40%) ethanol-exposed dams (P=0.03) and the litter size in the ongoing pregnancies ranged from 8-to-14. There was no inter-group difference for the litter size in the ongoing pregnancies by ANOVA analysis.

Chronic gestational exposure to ethanol causes intrauterine growth restriction. The pup weights of 3 control and 3 ethanol-exposed litters were documented at the time of tissue harvest. The mean weight of pups from control group was 0.473 ± 0.007 gram (Mean $\pm$ S.E.M.), whereas the mean weight of ethanol-exposed pups was significantly reduced (0.402 ± 0.012 gram; P=0.0008). The pup weights of dams that were on ad libitum chow diet (0.485 ± 0.005) were comparable to control liquid diet.

Chronic gestational exposure to ethanol alters placental development. H&E stained sections (5 μ m thick) of full thickness placentas with underlying mesometrial triangle were examined by light microscopy and subjected to image analysis using ImagePro Plus software. Two placentas per litter from 3 control (N=6) and 3 ethanol-exposed (N=6) dams were analyzed to generate the data. Since there were no morphological differences between the placentas of dams on chow and control liquid diet, only the placental weights of chow group were included in the analysis. The mean placental weight, which includes the underlying mesometrial triangle, of chow group (0.421 ± 0.009 gram; mean $\pm$ S.E.M.) and control liquid diet group (0.421 ± 0.013 gram) were comparable to each other. Although the mean placental weight was significantly increased in the ethanol group (0.465 ± 0.014 gram; P=0.02), histological studies demonstrated reduced placental thickness in ethanol-exposed (Fig. 1D) relative to control placentas (Fig. 1C). This abnormality was confirmed by image analysis, which demonstrated a significantly reduced mean placental thickness that included the labyrinthine and junctional zone in the ethanol-exposed relative to the control group (P=0.018; Fig. 1A). In contrast, the mean thicknesses of mesometrial triangle plus decidua basalis were similar in both groups (Figure 1B). Chronic gestational exposure to ethanol also produced several striking histopathological abnormalities including reduced populations of trophoblast giant cells [control 151 ± 12 ; ethanol 89 ± 13 (mean $\pm$ S.E.M.); P= 0.02] (Fig. 1F). Two ethanol-exposed placentas from the same litter had multifocal necrosis and parenchymal thrombi in the labyrinthine and spongiotrophoblast layers. In order to quantify the necrosis in these placentas, we outlined these foci and measured it using image analysis and compared it to the whole area of labyrinthine and spongiotrophoblast layers. The necrotic foci comprised 38% and 30% of the labyrinthine and spongiotrophoblast layers of these placentas. The mesometrial triangles of ethanol-exposed dams were significantly hypocellular (535 ± 69.7 ; mean $\pm$ S.E.M.) than control dams (886.2 ± 61.7 ; P=0.0036). Reduced or absent arterial transformation was noted in all the mesometrial triangles of ethanol-exposed dams and this effect involved all the vessels at the implantation site (Fig. 1H). To quantify this effect, vascular circumference was measured in control and ethanol-exposed mesometrial triangles. The mean vascular circumference in ethanol-exposed dams was 0.3 ± 0.02 mm (mean $\pm$ S.E.M.), which was significantly lower than the control dams (1.3 ± 0.06 mm; P=0.0005). The vascular muscular wall thickness of spiral arteries varied from 12 μ m to 14 μ m in ethanol-exposed dams, whereas the vascular muscular layer was completely disrupted in the control dams.

Chronic gestational exposure to ethanol alters gene expression in the insulin-IGF signaling pathways. Real-time quantitative RT-PCR detected mRNA transcripts encoding insulin, IGF-I and IGF-II polypeptide genes and insulin, IGF-I, and IGF-II receptors in both control and ethanol-exposed placentas with underlying mesometrial triangles. Ethanol-exposed placentas had significantly increased IGF-II and IGF-II receptor expression (Figs. 2B, 2E). In contrast, control and ethanol-exposed placentas had similar mean levels of IGF-I, IGF-I receptor, insulin receptor, and 18S rRNA (Figs. 2D, 2F, 2C).

The mRNA levels of IRS 1, 2, and 4 were examined since insulin and IGF-I transmit signals through IRS molecules to mediate growth, survival, and motility [28,29]. Since IRS-3 expression is restricted to rat adipocytes, its mRNA levels were not examined. IRS-1, IRS-2, and IRS-4 mRNA transcripts were detected in both control and ethanol-exposed placentas with underlying mesometrial triangles. In control placentas, IRS-2 was most abundant, followed by IRS-1, and then IRS-4 (Figs. 2G–2I). Chronic gestational exposure to ethanol significantly reduced mean mRNA levels corresponding to IRS-1 ($P=0.007$) and IRS-2 ($P=0.025$) in ethanol-exposed relative to control placentas (Figs. 2G–2H). In contrast, mean levels of IRS-4 were similar in control and ethanol exposed placentas (Fig. 2I).

Chronic gestational exposure to ethanol inhibits AAH expression

Placental tissue with underlying mesometrial triangle was analyzed for AAH mRNA and protein expression. Real time quantitative RT-PCR analysis demonstrated significantly reduced levels of AAH mRNA in ethanol-exposed relative to control placentas (Fig. 3A). Western blot analysis detected a single ~86 kD band corresponding to the expected size of AAH protein in control placentas, but not in ethanol exposed placentas (Fig. 3B, upper panel). Equal protein loading was demonstrated by re-probing the stripped membrane with antibodies to the p85 subunit of PI3 kinase (Fig. 3B, lower panel). The digital Western blot signals were quantified, and the relative levels of AAH and p85 were calculated. The mean calculated AAH/p85 ratio was significantly reduced in the ethanol-exposed relative to control placentas (Fig. 3C). Correspondingly, immunohistochemical staining of paraffin-embedded sections revealed strikingly reduced levels of AAH immunoreactivity in ethanol-exposed (Figs. 4C, 4D) relative to control placentas with underlying mesometrial triangles (Figs. 4A, 4B). For the most part, AAH immunoreactivity was localized in the cytoplasm of trophoblast and decidual stromal cells as previously described in human placenta [21].

Comparative analysis of AAH expression in the mesometrial triangle and placenta: Since the mesometrial triangle corresponds to the deep placental bed where the trophoblasts invade the uterus, the investigations were extended by examining AAH immunoreactivity in subdivided placental and mesometrial triangle tissue. To do this, at the time of harvesting, the placental tissue was separated from the underlying mesometrial triangle. The still adherent decidua basalis was scraped of the undersurface of placental disc. The mesometrial triangle was dissected from the uterus. The placental tissue was snap frozen separately and the mesometrial triangle and decidua basalis were snap frozen together for later analysis. Isolation of structures was verified by histological examination of a portion of each specimen. Real time quantitative RT-PCR analysis demonstrated significantly reduced levels of AAH mRNA in both regions, however the reduction was more striking in the mesometrial triangle (Fig. 5A). Western blot analysis and ELISA studies demonstrated higher levels of AAH expression in the mesometrial triangle compared with the placenta. Significantly reduced levels of AAH immunoreactivity in both the mesometrial triangle and placentas of ethanol-exposed relative to control dams were observed by ELISA studies (Fig. 5B).

DISCUSSION

This study demonstrates that chronic gestational exposure to ethanol causes increased fetal resorption, and impairs placental development and placentation. The increased fetal resorption corresponds with the reduced litter sizes observed previously in this experimental model of FAS [3,22]. Since ethanol present in maternal blood reaches both the fetus and placenta, its toxic effects on the fetus can be mediated either directly or indirectly. Direct effects of ethanol on fetal brain development have been well established using this model as well as by in vitro experimentation [2,3,22]. However, a new finding generated from these studies is that ethanol causes significant placental pathology especially within the labyrinthine layer, which is composed by syncytiotrophoblast, chorionic trophoblast, blood vessels and stroma, and is in direct contact with maternal blood. The reduced thickness of the placenta could be explained in part by increased necrosis, due to ischemia or infarction, as suggested by the presence of multi-focal parenchymal thrombi within the labyrinthine and spongiotrophoblast layers of ethanol-exposed placentas. Since nutrient exchange between the fetus and mother occurs within the labyrinthine layer, ethanol-induced reductions in the mass of this layer could impair nutrient delivery to the fetus, and thereby result in increased fetal demise as well as IUGR.

A second major placental abnormality associated with chronic gestational exposure to ethanol was reduced transformation of maternal uterine blood vessels within the mesometrial triangle, i.e. the deep placental bed. Transformation (remodeling) of uterine spiral arteries produces the high-flow, low-resistance circulation characteristic of intervillous space. Failure of maternal uterine vessels to transform from small muscular arteries to thin walled, distended, flaccid vessels compromises placental blood flow and nutrient exchange, and impaired uteroplacental blood flow has been linked to early pregnancy loss and IUGR [30]. Therefore, the motile and invasive properties of extravillous trophoblastic cells are critical for establishing and maintaining pregnancy, and ensuring adequate blood and nutrient delivery to the fetus to support growth and development.

In our experimental model of chronic gestational exposure to ethanol, which produces FASD in the pups [3,22], we detected significant increase in the expression levels of IGF-II and its receptor in ethanol-exposed placentas with underlying mesometrial triangle by quantitative RT-PCR. The IGF-I polypeptide gene expression and its receptor level in control and ethanol-exposed placentas with underlying mesometrial triangles were comparable. Since both insulin and IGF-I mainly activate PI3 kinase-Akt and Erk MAPK by transmitting signals downstream through IRS molecules [28], it was important to determine if IRS gene expression was altered by chronic ethanol exposure. Real time quantitative RT-PCR studies demonstrated significant reductions in placental IRS-1 and IRS-2 gene expression following chronic ethanol exposure. Therefore, expression of two major IRS molecules that transmit growth, survival, and metabolic signals in the placenta is impaired by chronic ethanol exposure. This suggests that, IGF-I and IGF-II activation of downstream growth and survival signaling mechanisms are impaired by ethanol. Since IGF-II can transmit growth and survival signals by activating PI3 kinase-Akt and Erk MAPK via its own receptor, or by binding to the insulin or IGF-I receptors, the increased levels of IGF-II and IGF-II receptor expression in ethanol-exposed placentas may represent a compensatory response to impairments in IGF-I signaling mediated by IRS-I and IRS-2 depletion.

The fourth important finding in this study was that ethanol inhibited the expression of AAH, which is a downstream target of insulin and IGF signaling, and an important mediator of cell motility and invasiveness which are required for successful placentation. We have previously demonstrated that the extravillous trophoblastic cells that mediate the placentation and vascular transformation are strongly immunoreactive for AAH as well as cytokeratin 7, which is a trophoblastic cell marker [21]. The studies revealed significantly reduced AAH expression at

both the mRNA and protein levels in ethanol-exposed placentas, as demonstrated by real time quantitative RT-PCR, Western blot analysis with digital densitometry, immunohistochemical staining, and ELISA. Previous studies showed that insulin/IGF stimulation of AAH is mediated by activation of PI3 kinase-Akt or Erk MAPK [17,20], and that ethanol inhibits insulin/IGF activation of these kinases [22,31,32]. Therefore, the reduced levels of AAH protein observed in ethanol-exposed placentas were likely mediated by impaired signaling through PI3 kinase-Akt and/or Erk MAPK leading to reduced levels of AAH mRNA. Since AAH has an important role in motility and invasion, ethanol-mediated inhibition of the AAH gene most likely represents an important mechanism of ethanol-impaired placentation, resulting in increased fetal demise and IUGR. The higher levels of AAH detected in the mesometrial triangle compared with the labyrinthine region, and significantly reduced AAH expression in the mesometrial triangle region of ethanol-exposed placentas which exhibited impaired placentation are consistent with our hypothesis that AAH has an important role in placental development and therefore fetal growth and maintenance.

IUGR is an important feature of FASD, but its pathogenesis is not well understood. IGFs, especially IGF-II, are critical regulators of fetal growth and development [8,9,33,34], but they also mediate placentation and placental function [10]. Extravillous trophoblasts express IGF-II, with the highest levels at the invading front [35]. IGF II stimulates trophoblast invasion [36], whereas reduced placental expression of IGF-II is associated with IUGR [37]. Therefore, impaired function of extravillous trophoblasts may represent a major cause of IUGR [38]. In our experimental FAS model, we demonstrated ethanol-induced IGF-II and IGF-IIR mRNA levels. Although the precise mechanism has not yet been determined, potential explanations for increased IGF-II levels include reduced bioavailability as a result of increased IGF-IIR levels or a compensatory response to ethanol inhibition of downstream IGF-I signaling. IGF-II signaling pathway is not fully characterized but currently under investigation and therefore additional future experiments will be required to fully divulge the mechanism of ethanol-impaired placentation. Though further studies are required, we hypothesize that ethanol inhibition of IGF signaling through IRS molecules, which were markedly reduced in our model, resulting in reduced levels of AAH expression in extravillous trophoblasts impairs functions required for placentation, and thereby contributes to the frequent occurrence of IUGR in FASD.

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Figure 1.

Effects of chronic gestational exposure to ethanol on placental morphology. Image analysis was used to measure (A) placental and (B) mesometrial triangle thickness in control and ethanol-exposed dams. Graphs depict the mean $\pm$ SEM of measurements and inter-group comparisons were made using Student T-tests. Significant P-values are indicated over the graphs. (C–H) Paraffin-embedded histological sections of placenta (N=2 per litter) from 3 control (C, E, G) and 3 ethanol-exposed (D, F, H) dams were stained with H&E and examined by light microscopy. (C, D) Low magnification images show effects of ethanol on placental thickness (arrows delineate the labyrinthine and junctional zones, original magnification x40). (E–H) Higher magnification images illustrate trophoblast giant cells at the maternal-fetal

interface (arrows in E and F; original magnification x200) and spiral maternal arteries in the mesometrial triangle (G, H; original magnification x400).

Figure 2.

Chronic gestational exposure to ethanol alters gene expression in the insulin-IGF signaling pathways. RNA extracted from control and ethanol-exposed placentas with underlying mesometrial triangle (N=4 per litter; 2 dams per group) was reverse transcribed using random oligodeoxynucleotide primers. Gene expression corresponding to (A) IGF-I, (B) IGF-II, (D) IGF-I receptor, (E) IGF-II receptor, (F) insulin receptor, (G) IRS-1, (H) IRS-2, and (I) IRS-4 mRNA transcripts, and (C) 18S ribosomal (r) RNA was measured by real-time quantitative RT-PCR using gene-specific primers. The values were normalized to 18S rRNA. Graphs depict the mean $\pm$ SEM of measurements and inter-group comparisons were made using Student T-tests. Significant P-values are indicated over the graphs.

Figure 3.

Chronic ethanol exposure inhibits AAH expression in placental tissue. (A) AAH mRNA levels were measured in placenta with underlying mesometrial triangle by real time quantitative RT-PCR (see Methods and Figure Legend 2). (B, upper panel) AAH protein expression levels were examined by Western blot analysis. Immunoreactivity was detected with A85G6 monoclonal antibody. (B, lower panel) As a loading control, the blots were stripped and reprobed with polyclonal antibody to p85 (subunit of PI3 kinase). Immunoreactivity was revealed with horseradish peroxidase conjugated secondary antibody and ECL reagents. (C) Immunoreactivity was quantified by digital imaging and the results corresponding to the mean

levels ($\pm$ SEM) of the AAH/p85 ratios are depicted graphically. Inter-group comparisons were made using Student T-tests and the P-value is shown over the graph.

Figure 4.

Chronic gestational exposure to ethanol reduces AAH immunoreactivity in trophoblast. Paraffin-embedded histological sections were immunostained with FB50 monoclonal antibody to AAH. Low magnification (original, x40) images of (A) control and (C) ethanol-exposed placentas with underlying mesometrial triangle depict relative levels of AAH immunoreactivity. High magnification (original, x400) images illustrate relative levels of AAH immunoreactivity in the mesometrial triangle of (B) control and (D) ethanol-exposed placentas. Insets in panels B and D show higher magnification images (original, x800).

Figure 5.

Effects of ethanol on AAH expression in the mesometrial triangle and placenta. RNA and protein were extracted from separated placenta and mesometrial triangle with decidua basalis, and AAH expression was measured by (A) real-time quantitative RT-PCR (see Methods and Figure 2 legend) or (B) ELISA. For ELISA, AAH immunoreactivity was detected with the A85E6 monoclonal antibody. Monoclonal antibody to Hepatitis B virus surface antigen (HBVSAg) was used as a negative control. Immunoreactivity detected with HRP-conjugated secondary antibody and chemiluminescence reagents was measured in a TopCount machine (arbitrary relative light units; RLU). The graphs depict the mean $\pm$ SEM levels of specific AAH immunoreactivity (HBVSAg immunoreactivity subtracted) in mesometrial triangle and

placental tissue of control and ethanol-exposed dams. Inter-group comparisons were made using Student T tests. Significant P-values are indicated above the graphs.

TABLE 1

Primer pair sequences for real-time quantitative RT-PCR*

Primer		Sequence (5'→3')	Position (mRNA)	Amplicon size (bp)
18S	For	GGA CAC GGA CAG GAT TGA CA	1278	50
	Rev	ACC CAC GGA ATC GAG AAA GA	1327	
AAH	For	TGC CTG CTC GTC TTG TTT GTG	666	118
	Rev	ATC CGT TCT GTA ACC CGT TGG	783	
IN	For	TTC TAC ACA CCC AAG TCC CGT C	433	135
	Rev	ATC CAC AAT GCC ACG CTT CTG C	567	
IN-R	For	TGA CAA TGA GGA ATG TGG GGA C	875	129
	Rev	GGG CAA ACT TTC TGA CAA TGA CTG	1003	
IGF-I	For	GAC CAA GGG GCT TTT ACT TCA AC	65	148
	Rev	TTT GTA GGC TTC AGC GGA GCA C	212	
IGF-IR	For	GAA GTC TGC GGT GGT GAT AAA GG	2116	135
	Rev	TCT GGG CAC AAA GAT GGA GTT G	2250	
IFG-II	For	CCA AGA AGA AAG GAA GGG GAC C	763	116
	Rev	GGC GGC TAT TGT TGT TCA CAG C	878	
IGF-IIR	For	TTG CTA TTG ACC TTA GTC CCT TGG	1066	114
	Rev	AGA GTG AGA CCT TTG TGT CCC CAC	1179	
IRS-1	For	GAT ACC GAT GGC TTC TCA GAC G	604	134
	Rev	TCG TTC TCA TAA TAC TCC AGG CG	737	
IRS-2	For	CAA CAT TGA CTT TGG TGA AGG GG	255	109
	Rev	TGA AGC AGG ACT ACT GGC TGA GAG	363	
IRS-4	For	ACC TGA AGA TAA GGG GTC GTC TGC	2934	132
	Rev	TGT GTG GGG TTT AGT GGT CTG G	3065	

* AAH: aspartyl-(asparaginyl) β -hydroxylase; IN: insulin; INR: insulin receptor; IGF-I: insulin-like growth factor-I; IGF-IR: IGF-I receptor; IGF-II: insulin-like growth factor-II; IGF-IIR: IGF-II receptor; IRS: insulin receptor substrate; For: forward primer; Rev: reverse primer; Position: initial nucleotides for primer binding; bp: base pair size of amplicon.