Acute ethanol exposure in pregnancy alters the insulin-like growth factor axis of fetal and maternal sheep

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Gatford KL, Dalitz PA, Cock ML, Harding R, Owens JA. Acute ethanol exposure in pregnancy alters the insulin-like growth factor axis of fetal and maternal sheep. Am J Physiol Endocrinol Metab 292: E494–E500, 2007. First published September 26, 2006; doi:10.1152/ajpendo.00269.2006.—Maternal ethanol intake during pregnancy impairs fetal growth, but mechanisms are not clearly defined. Reduced IGF abundance or bioavailability in the fetus and/or mother may contribute to this growth restriction. We hypothesized that an episode of acute ethanol exposure, mimicking binge drinking would restrict fetal growth and perturb the maternal and fetal IGF axes. Pregnant sheep were infused intravenously with saline or ethanol (1 g/kg maternal wt) over 1 h, on days 116, 117, and 118 of gestation (start of 1st infusion = time 0, term is 147 days). Maternal and fetal plasma IGF and IGF-binding protein (IGFBP) concentrations were measured before and after each infusion. Compared with controls, ethanol exposure reduced fetal weight at day 120 by 19%, transiently reduced maternal plasma IGF-I (−35%) at 30 h, and decreased fetal plasma IGF-II (−28%) from 24 to 54 h after the first infusion. Ethanol exposure did not alter maternal or fetal plasma concentrations of IGFBP-2 and IGFBP-3, measured by Western ligand blotting. We conclude that suppression of maternal and fetal IGF abundance may contribute to fetal growth restriction induced by acute or binge ethanol exposure.

SEVERE AND CHRONIC EXPOSURE of the fetus to ethanol leads to distinctive craniofacial and central nervous system abnormalities and lifelong learning and behavioral difficulties (43). Another major feature of chronic gestational ethanol exposure is restricted fetal growth, which is seen in humans (2, 11) and rats (1, 3, 31); this growth restriction persists after birth in both species (1, 5, 7, 10). However, the mechanistic basis for the restriction of fetal growth in response to ethanol exposure is largely unknown.

The IGF axis is important for growth before and after birth. The effects of deletion of the IGF-I or -II gene in mice indicate that IGF-I is most critical for fetal and postnatal growth, whereas IGF-II regulates placental and fetal, but not postnatal, growth (4, 13). Consistent with their roles in growth regulation, fetal and neonatal plasma IGF-I and IGF-II concentrations are usually reduced in human intrauterine growth restriction (IUGR) (25, 27, 28) as well as in experimental IUGR, such as chronic undernutrition in rats (44) and acute severe maternal undernutrition (6) and restricted placental implantation (33) in sheep. Plasma IGF-binding protein-3 (IGFBP-3) is also increased in the late-gestation IUGR human fetus (25), which might further decrease IGF bioavailability.

Studies of chronic ethanol exposure have implicated altered fetal and maternal circulating IGFs as causes of growth restriction. The one human study to date found a 35% decrease in serum IGF-I in neonates with clinically defined FAS compared with nonexposed neonates (18). Neonates exposed in utero to ethanol, but who were without clinical signs of FAS, had normal serum IGF-I (18), probably reflecting lower levels of ethanol exposure (17). Maternal serum IGF-I just before delivery was not altered by ethanol consumption in this study (18). In rats, variable effects of ethanol exposure during gestation have been reported for fetal plasma concentrations of IGF-I (−22% or unchanged), IGF-II (−13% trend or +32%), and IGFBP (−20% total IGFBP or 44–55% decrease in individual IGFBP bands) (31, 39). Maternal ethanol treatment throughout pregnancy also reduces IGF gene expression (14, 36, 39) and/or secretion (30) by near-term fetal rat liver, brain, heart, kidney, and placenta.

Changes in the maternal IGF axis might also impair fetal growth via its effects on maternal metabolism and placental function. Ethanol ingestion throughout pregnancy in rats halved maternal post partum plasma IGF-I, doubled IGF-II, and reduced IGFBP-3 by ~40% (8), suggesting that decreased production or bioavailability of IGF-I might have contributed to impaired fetal growth. In humans, ethanol ingestion often occurs acutely (i.e., in binges) rather than chronically; however, effects of acute ethanol exposure on the maternal and fetal IGF axes and their relation to fetal growth have not been investigated. Our aim was to investigate the role of the IGF axes in ethanol-induced fetal growth restriction by determining the effects of an episode of acute ethanol exposure on fetal growth and components of the maternal and fetal IGF axes.

MATERIALS AND METHODS

Animals. All procedures were approved by the Animal Welfare Committee of Monash University. By use of established techniques, 12 twin-bearing pregnant sheep (Merino × Border Leicester) were anesthetized with halothane (2% in oxygen) and underwent aseptic surgery at 110 ± 1 days of gestation (term ~147 days). Polivynil catheters were inserted into a femoral artery and vein and into the amniotic sac of each fetus and into a carotid artery and jugular vein of each ewe. Antibiotics (penicillin and dehydro-streptomycin, 0.5 ml; Troy Laboratories, Smithfield, Australia) were injected intramuscularly into the fetus at surgery. Following recovery from surgery, ewes were housed individually with 12:12-h light (0700–1900)-dark cy-
cles. Ewes were fed 1.2 kg of lucerne chaff at 0830, and if this was consumed by 1700 additional feed was provided to ensure ad libitum food availability. Water was available ad libitum.

Protocol. Commencing at 116 ± 1 days of gestation, ewes were infused for 1 h with 40% ethanol in saline (1 g ethanol·kg maternal wt⁻¹·h⁻¹ iv, ethanol-treated group, n = 7) or with saline (control group, n = 5) between 0930 and 1030 on three consecutive days. The start of the first infusion is regarded as time 0. On each treatment day, fetal and maternal arterial blood samples (~2 ml) were taken prior to and at 1, 2, 3, 4, and 6 h after the commencement of ethanol infusion.

Blood alcohol concentration (BAC) was measured by automated enzymatic analysis (Dade Behring Diagnostics, Sydney, Australia) in unfrozen plasma at each time point. Remaining plasma was stored at −20°C for later analyses. Fetal blood cells were reinfused into the fetus to maintain packed cell volume. Fetal arterial blood gas concentrations were monitored, and fetuses were not hypoxic or hypercapnic at any time. At 120 days gestation, ewes and fetuses were euthanased by intravenous administration of an overdose of pentobarbitone sodium. Fetuses were dried and weighed.

Measurement of plasma IGF-I and IGF-II. IGF-I and -II concentrations were measured in plasma from each ewe and one of its fetuses (randomly chosen) at 0, 1, 2, 3, 4, 6, 24, 30, 48, and 54 h after the start of the first ethanol infusion. Plasma IGF-I and -II were measured by RIA after separation of IGF and IGFBP by size exclusion HPLC under acidic conditions (34). Maternal and fetal plasma samples were injected onto separate HPLC columns dedicated to ovine postnatal or maternal samples. Four fractions of eluate (fraction 1, containing IGFBP; fraction 2, interpeak; fraction 3, containing IGF; and fraction 4, postpeak) were routinely collected for each acidified plasma specimen, using times determined for each column based on elution times of 125I-labeled IGF-I and IGF immunoactivity. Recovery of 125I-IGF-I in three HPLC runs containing maternal plasma was 79.8 ± 0.9% and in 13 HPLC runs containing fetal plasma was 87.1 ± 0.7%.

Plasma IGF-I concentrations were measured by analysis of neutralised HPLC fraction 3, in a RIA specific for IGF-I (15), using the Conlon rabbit polyclonal antibody to human IGF-I. Samples were assayed in triplicate. Inter- and intra-assay CVs for a fraction 3 eluate pool containing 150 ng/ml IGF-I were 5.9% and 6.4% respectively (n = 14 assays). Covariance for extraction and assay of a fetal plasma quality control (QC) containing 165 ng/ml IGF-I, and included in each HPLC run of fetal samples, was 10.1% (n = 13 HPLC runs). Covariance for extraction and assay of a maternal plasma QC containing 217 ng/ml IGF-I and included at the start, middle, and end of each HPLC run of maternal samples was 6.4% (n = 3 HPLC runs, 9 measurements).

Plasma IGF-II concentrations were measured by analysis of HPLC fraction 3 in an RIA specific for IGF-II (9). Samples were assayed in triplicate. Inter- and intra-assay CVs for a fraction 3 eluate pool containing 95 ng/ml IGF-II were 15.8 and 4.1%, respectively (n = 8 assays). Covariance for extraction and assay of a fetal plasma QC containing 1.021 ng/ml IGF-II, and included in each HPLC run of fetal samples, was 9.0% (n = 13 HPLC runs). Covariance for extraction and assay of a maternal plasma QC containing 681 ng/ml IGF-II and included at the start, middle, and end of each HPLC run of maternal samples was 9.3% (n = 3 HPLC runs, 9 measurements).

Measurement of plasma IGFBP. IGFBP concentrations were measured in the same animals as for IGF, in plasma collected before and then 6 h after the start of each infusion, i.e., at 0, 6, 30, and 54 h. Plasma samples (20 μl of a 1:10 dilution) were heated to 65°C for 20 min and then subjected to nonreducing discontinuous SDS-PAGE on a 4.4% stacking gel and 10% separating gel. Proteins were electrotransfered to 0.2-μm nitrocellulose and air-dried overnight, and IGFBP was detected by Western blotting with 125I-IGF-II (23). IGFBP bands were visualized by exposing membranes to X-ray film at −80°C for ~30 days with intensifying screens. 14C protein Mr markers (Rainbow 14C methylated protein markers; Amersham Life Science UK) and a pregnant sheep QC plasma, prepared as above, were included in each gel. Autoradiographs were scanned densitometrically (Gel Doc scanner; Bio-Rad Laboratories), and the density of each band was expressed relative to the mean density of the two IGFBP-3 bands of the pregnant ewe QC plasma. IGFBP bands present in ovine plasma have been previously identified as IGFBP-3 (42- to 50-kDa doublet), IGFBP-2 (33 kDa), IGFBP-1 (~28 kDa), and IGFBP-4 (24 kDa) (9, 22).

Statistical analysis. Weights of control and ethanol-treated fetuses at 120 days gestation were compared by one-way ANOVA; only fetuses with IGF measurements were included in this analysis. Maternal and fetal BAC were compared in ethanol-infused animals by paired-sample t-test at each time. Plasma IGF concentrations were analyzed both as absolute concentrations and as a percentage of the preinfusion values to reduce interanimal variability. Effects of time and ethanol treatment on plasma IGF and total IGFBP throughout the study and during the last 2 days of the study only, and on plasma abundance of IGFBP-2 and IGFBP-3 throughout the study, were analyzed by mixed-model analysis in SPSS v.13.0. A P value of <0.05 was taken as significant, and data are reported as means ± SE unless otherwise indicated.

RESULTS

Model characteristics. All ewes consumed ~1.2 kg of feed daily throughout the study. BAC in the ewe and fetus peaked at ~0.10–0.11 g/dl at the end of ethanol infusions and then fell rapidly to ~0.04 g/dl at 6 h and were undetectable 24 h after the start of each infusion (Fig. 1). BAC was nondetectable in saline-infused ewes and fetuses at all times. Within the ethanol-infused group, BAC of the ewe and her fetus were strongly positively correlated at the majority of measurements taken after each infusion (data not shown). The BAC of the fetus exceeded that of the ewe after the first infusion (at 1, 2, and 3 h), after the second infusion (at 26 h), and after the third infusion (at 49 and 54 h).

Fetal weight. At autopsy, ethanol-exposed fetuses were 19% lighter than controls (P = 0.034; control fetuses: 2.70 ± 0.19 kg; ethanol-exposed fetuses: 2.20 ± 0.11 kg).

Fig. 1. Blood alcohol concentration in control (n = 5, ●) and ethanol-treated (n = 7, ○) ewes and fetuses. Infusion periods are shaded. Data are presented as means ± SE.
Plasma IGF-I and IGF-II. Preinfusion maternal and fetal plasma concentrations of IGF-I and IGF-II did not differ between groups ($P > 0.1$ for all).

Absolute concentrations of IGF-I in maternal plasma (Fig. 2) changed with time ($P = 0.021$) but did not differ between groups ($P = 0.38$). When expressed relative to preinfusion concentrations, maternal plasma IGF-I (Fig. 2) varied differently with time in control and ethanol-treated groups ($P = 0.009$); maternal plasma IGF-I increased gradually from baseline in control ewes but changed little in ethanol-treated ewes. The effect of treatment on maternal plasma IGF-I was therefore evaluated separately at each time. The relative concentration of maternal plasma IGF-I was 35% lower in ethanol-treated than in control ewes at 30 h ($P = 0.002$) but tended to be higher at 2 h (15%, $P = 0.067$). Absolute concentrations of IGF-II in maternal plasma (Fig. 2) changed with time ($P = 0.042$) and tended to change differently with time in each group ($P = 0.054$) and were therefore evaluated separately at each time. Absolute concentrations of IGF-II in maternal plasma tended to be lower in ethanol-treated than in control ewes at 6 h (21% lower, $P = 0.074$), 48 h (26% lower, $P = 0.054$), and at 54 h (26% lower, $P = 0.056$). The relative concentration of maternal plasma IGF-II (Fig. 2) also changed differently with time between groups ($P = 0.045$) and was, or tended to be, lower in ethanol-treated than in control ewes at 4 h (24% lower, $P = 0.013$), 30 h (36% lower, $P = 0.036$), 48 h (41% lower, $P = 0.098$), and 54 h (42% lower, $P = 0.032$).

Absolute and relative concentrations of IGF-I in fetal plasma (Fig. 3) did not change with time or between groups ($P > 0.5$ for each). Absolute concentrations of IGF-II in fetal plasma (Fig. 3) tended to change differently with time in ethanol-treated and control fetuses ($P = 0.095$) but were not different between treatments at any single time. Relative concentrations of IGF-II in fetal plasma (Fig. 3) tended to change with time.
and were reduced by maternal ethanol treatment overall ($P = 0.034$) and changed similarly with time in both groups ($P = 0.107$). In the last 2 days of treatment only, relative concentrations of fetal plasma IGF-II were 28% lower in ethanol-treated than in control fetuses ($P = 0.006$).

**Plasma IGFBP profiles.** Five IGFBP bands were visible on the Western ligand blots, as shown for pooled maternal (Fig. 4A) and fetal plasma (Fig. 4B) from control and ethanol-treated animals. IGFBP-3 (42- to 50-kDa doublet) and IGFBP-2 (33-kDa band) were visible in all individual samples. The IGFBP-1 and IGFBP-4 bands were visible for some, but not all, samples and were not quantified.

IGFBP-3 abundance in maternal plasma (Fig. 5) tended to increase with time ($P = 0.098$) but did not differ with treatment ($P = 0.60$) or change differently with time between treatments ($P = 0.22$). IGFBP-3 abundance was higher in maternal than in fetal plasma (Fig. 5). IGFBP-2 abundance in maternal plasma (Fig. 5) did not vary with time or treatment ($P > 0.4$ for each). Maternal plasma IGFBP-3 and IGFBP-2 expressed relative to their preinfusion abundances were also unaffected by time or treatment ($P > 0.3$ for all).

IGFBP-3 abundance in fetal plasma (Fig. 5) did not vary with time ($P = 0.84$) or treatment ($P = 0.17$) or change differently with time between treatments ($P = 0.19$). IGFBP-2 abundance in fetal plasma (Fig. 5) did not vary with time ($P = 0.17$) or treatment ($P = 0.87$) or change differently with time between treatments ($P = 0.75$). Fetal plasma IGFBP-3 and IGFBP-2 expressed relative to their preinfusion abundances were also unaffected by time or treatment ($P > 0.16$ for all).

**DISCUSSION**

Acute exposure to ethanol on three successive days at ~0.8 of gestation caused an apparent cessation of fetal growth. Maternal ethanol treatment also transiently reduced relative concentrations of IGF-I in maternal plasma by 35% after the second exposure compared with control, reduced relative concentrations of IGF-II in maternal plasma by ~40% in samples collected after the second exposure, and decreased relative concentrations of IGF-II in fetal plasma by ~30% from 24 to 54 h after the first exposure. It therefore appears that repeated acute ethanol exposure, like chronic ethanol exposure, impairs fetal growth in conjunction with changes in the maternal as well as fetal IGF axes.

Maternal ethanol exposure appeared to completely stop fetal growth during and for 2 days after ethanol infusions. Previously published growth curves for Merino sheep fetuses show a weight gain of ~18% between 116 and 120 days of gestational age (29), equivalent to a weight gain of 421 g based on the post mortem weights of control fetuses in the present study. The 503-g difference in fetal weight between the groups implies that fetuses in the ethanol-treated group did not grow over the 5-day period from the first ethanol infusion to postmortem. We do not yet know when or whether fetal growth resumes after ethanol exposure ceases. Chronic ethanol consumption in pregnant women reduces birth weight in a dose-dependent manner, particularly when ethanol consumption...
occurs or continues in the third trimester, with 190- to 509-g decreases in birth weight reported following chronic ethanol abuse (2, 11, 24). Observations in the rat are consistent with the hypothesis that fetal growth is most susceptible to ethanol in late gestation; exposure during only the last third trimester of pregnancy reduced birth weight to a similar extent as treatment throughout all of pregnancy, whereas treatment during the first or second weeks only did not substantially reduce birth weight (1). Little information is available on the effects of acute ethanol consumption during pregnancy in women. Occasional binge drinking (~5 standard drinks on a single occasion) in the month before pregnancy or during the first two trimesters did not reduce the birth size of infants born to women who drank less than one standard drink per day throughout pregnancy (42), although it remains possible that fetal growth was acutely impaired at the time of exposure. In the present study, peak BAC was ~0.10 g/dl in the ewe and fetus at the end of each infusion, implying that even a brief exposure to alcohol levels similar to those reached in human binge drinking (32) severely restricts fetal growth. The sensitivity of fetal growth to ethanol may vary between species, as maternal BAC of ~0.1 g/dl did not reduce birth weight in chronically exposed rats (3). Twin fetuses may also be more susceptible to growth retardation than singletons, because their supply of nutrients is restricted prior to administration of any challenge (such as ethanol). Whether fetal growth retardation induced by acute or binge ethanol exposure is transient, and the extent to which this might be followed by catch-up growth in utero, is not yet known, as longitudinal data on fetal size during and following acute ethanol exposure are not currently available. Similarly, effects of acute ethanol exposure on postnatal growth and development are largely unknown.

Although fetal growth was profoundly retarded by repeated acute maternal and fetal ethanol exposure, we observed less marked changes in the maternal and fetal ovine IGF axes. The power of the study was sufficient to detect decreases of 30–40% in plasma IGF, which we expected on the basis of our previous studies of IGF abundance in fetal growth restriction in this species (33). Ethanol exposure transiently reduced relative maternal plasma IGF-I by 35%, consistently reduced (by ~40%) relative maternal plasma IGF-II, and tended to reduce (by ~22%) absolute maternal plasma IGF-II after the second exposure. This is the first report showing that ethanol reduces maternal IGFs during pregnancy in any species and is consistent with reported reductions in maternal plasma IGF-I but contrasts with reported increases in maternal plasma IGF-II postpartum in the rat following chronic ethanol treatment (8). The differences in maternal IGF-II between the treatment groups in this study largely reflected steady increases in IGF-II in control ewes, which were apparently prevented by ethanol treatment. This increase is unlikely to be related to postsurgery recovery, since surgery occurred 6 days prior to the start of infusions. IGF-II is expressed in multiple tissues (liver, lung, skeletal muscle, adrenal gland, and kidney) in the nonpregnant adult sheep (26), but maternal tissue expression during late pregnancy has not yet been characterized, and the present study is the first to report circulating levels during this period. Circulating IGF-I in maternal plasma rises during pregnancy (16) and may serve as a marker for maternal metabolic adaptations to pregnancy that increase the availability of nutrients to the fetus. The difference in maternal IGF-I between the two groups was only transient and reflected increasing IGF-I in control ewes as well as decreasing IGF-I in ethanol-treated ewes. The transient nature of this difference in maternal IGF-I suggests that repeated acute ethanol exposure is unlikely to impair maternal metabolic adaptations to pregnancy. Nutrient supply to the fetus may nevertheless be profoundly compromised and contribute to fetal growth retardation in this model due to the effects of ethanol on placental function. Ethanol impairs placental uptake and/or delivery to the fetus of glucose and amino acids in rats and humans (20, 21, 40, 41). Ethanol may also impair fetal tissue uptake of nutrients from the fetal circulation, for example by decreasing the expression and activity of glucose transporters. Ethanol administration to adult rats rapidly decreases plasma membrane abundance of GLUT1 and GLUT3 in the cerebral cortex (19), and GLUT1 expression and in vivo and in vitro glucose uptake are reduced in brain tissue of ethanol-exposed fetal rats (37, 38). These studies suggest that both impaired placental transport of nutrients and impaired fetal tissue uptake of nutrients may contribute to fetal growth restriction caused by acute ethanol exposure.

The fetal IGF axis was suppressed by repeated acute ethanol exposure to a greater extent than the maternal IGF axis. Relative fetal plasma IGF-II was decreased by 28% over the final 2 days of ethanol exposure, reflecting an apparent increase in plasma IGF-II from preinfusion levels in control fetuses and a concurrent decrease in plasma IGF-II in ethanol-treated fetuses. In contrast, chronic ethanol exposure in rats increased fetal plasma IGF-II in late gestation by 32% in one study (31), and tended to decrease (~13%) fetal plasma IGF-II in another (39). The mechanisms responsible for decreased circulating IGF-II in the present study are unknown. Multiple fetal tissues as well as placenta synthesize IGF-II in the fetal sheep. Chronic ethanol exposure decreased IGF-II gene expression in fetal rat brain and liver, although not lung or placenta (14, 36, 39), but effects of acute exposure have not been investigated. In the present study, concentrations of IGFBP-2 and IGFBP-3 did not differ significantly between treatment groups, reflecting significant interindividual variation.

In contrast with suppression of fetal and neonatal plasma IGF-I following chronic ethanol exposure in the rat (39) and human (18), repeated acute ethanol exposure did not change fetal plasma IGF-I in sheep in the present study. This may reflect the acute rather than chronic treatment or that changes in plasma IGF-I may develop over a longer time period. Despite the moderate effects on circulating IGF abundance, acute ethanol treatment probably impairs the anabolic effects of circulating IGFs on fetal growth promotion by direct effects at the receptor level. Chronic ethanol exposure from day 6 of pregnancy to term, with maternal BACs of 0.35 to 0.45 g/dl, reduces the receptor tyrosine kinase activities of the insulin and type I IGF receptor in cerebellum of newborn rat pups and is hypothesized to contribute to neuronal loss in this model (12). Ethanol also impairs IGF-I and IGF-II signaling via the type 1 IGF receptor in vitro, decreasing type 1 receptor autophosphorylation and blocking cell-proliferative responses to either IGF at concentrations of only 0.05 to 0.12 g/dl (35); we therefore hypothesize that peak BAC of ~0.10 to 0.11 g/dl in our study inhibits IGF-promotion of fetal (and perhaps placental) growth through the type I IGF-receptor.

We conclude that suppression of both maternal and fetal IGF abundance may contribute to fetal growth restriction induced
by repeated acute or binge ethanol exposure. Further investigations are needed to define the mechanisms responsible and to assess the long-term effects of binge ethanol exposure on growth and the IGF axes in fetal and postnatal life.

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