Ethanol-Induced Changes in Insulin-Like Growth Factors and IGF Gene Expression in the Fetal Brain (44025)

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Abstract. **Brain growth retardation is a major feature of the fetal alcohol syndrome (FAS). Insulin-like growth factors (IGF-I and IGF-II) have been shown to exert significant metabolic and growth-promoting effects. Previously, we showed that circulating levels of IGF-I as well as hepatic gene expression of both IGFs were decreased in newborn offspring of rats fed ethanol during pregnancy. This study investigated the effects of maternal ethanol ingestion on fetal rat brain growth and on levels of IGF-I and IGF-II, as well as their mRNAs, in fetal brain. IGF-binding protein (IGFBP) levels also were determined. Rats were fed 5% w/v ethanol in a liquid diet during gestation (EF group). Weight-matched animals were pair-fed equicaloric control diet (PF group) or were fed *ad libitum* (AF group). The mean fetal brain weight of EF offspring was 13% and 16% lower ($P < 0.01$) than that of PF and AF offspring, respectively. Body weight of EF pups was decreased to a greater extent, resulting in higher brain to body weight ratios in EF pups than in either control group ($P < 0.05$). IGF-I levels in EF pups decreased by 33% and 41% compared with the corresponding PF and AF values ($P < 0.01$). IGF-I mRNA levels decreased by 27% and 40% compared with PF and AF values, respectively. A positive correlation was observed between brain IGF-I level and brain weight ($r = 0.561$, $P < 0.01$). IGF-II levels were not affected despite a 50% decrease in IGF-II expression. In PF animals, the fetal brain IGF-I and IGF-II mRNA levels were reduced by 28% and 21%, apparently in response to undernutrition. IGF-binding proteins levels were low in the EF group but not statistically significant compared with control values. The diminished fetal brain concentration of IGF-I and decreased gene expression of IGFs may play a role in brain growth retardation associated with FAS.** [P.S.E.B.M. 1996, Vol 212]

Fetal brain growth retardation is a major feature of the fetal alcohol syndrome (FAS). In humans and animals, prenatal ethanol exposure is associated with fetal brain weight reduction and multiple aberrations of the development and maturation of the central nervous system (1). The mechanism of the teratogenic effects of ethanol is unknown. However, mechanisms proposed include impaired placental transport, fetal hypoxia, altered prostaglandin metabolism, and malnutrition, as well as altered fuel-hormone homeostasis, and abnormal protein synthesis (2, 3). The actual mechanism is probably multifactorial, with the contributions of different factors specific to developmental stages and to the type of tissue affected.

Insulin-like growth factors (IGF-I and IGF-II) are mitogenic peptides which are structurally related to proinsulin and which exert significant effects on cell metabolism and growth (4). Expression of the genes for both these peptides is subject to precise developmental and tissue-specific regulation. This is true as well for the specific binding proteins and receptors which modulate and mediate their actions (5, 6). It has been suggested that IGFs, acting in an autocrine, para-

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crine or endocrine fashion, exert multiple biological actions on neural cells which influence the development and differentiation of the CNS (7).

Intrauterine growth retardation, induced by maternal fasting or protein deprivation, correlates with fetal serum IGF-I and IGF-II levels and their gene expression (8–10). Fetal rat liver and serum IGF-I levels are decreased and correlate with body weight in growth retardation induced by uterine artery ligation (11). Breese *et al.* (12) reported a decrease in plasma IGF-I but not IGF-II levels in growth-retarded offspring of ethanol-fed rats studied at birth and postnatally. However, Mauceri *et al.* (13) found no effect on plasma IGF-I but increased IGF-II in rat fetuses exposed to ethanol. In agreement with the former study, we previously observed decreased plasma IGF-I levels and reduced levels of liver IGF-I and IGF-I mRNA in term fetal rats after exposure to ethanol *in utero* (14). Furthermore, serum IGF-I levels correlated with fetal body weight. The present study analyzed IGF-I and IGF-II gene expression and IGF levels in the fetal brain of the same animals. The data show a decrease in fetal brain gene expression of IGF-I, as well as decreased IGF-I concentration, following prenatal ethanol exposure. Fetal brain IGF-II mRNA expression was reduced and accompanied by a trend toward a decrease in IGF-II level.

Materials and Methods

Animals and Diet. Details of animal preparation have already been described (14). Briefly, on Day 1 of pregnancy, 8-week-old Sprague-Dawley rats were weight matched and assigned at random to one of the three treatment protocols. The experimental animals (EF group) received 5% w/v ethanol in liquid diet *ad libitum* throughout gestation. Other groups were given an ethanol-free equicaloric liquid diet equal in quantity to that consumed by each EF dam (PF group) or *ad libitum* (AF group). The diet formulation has been described previously. Diet ingredients were obtained from Dyets Inc, (Bethlehem, PA). Food intake and body weight were monitored at regular intervals. The animals did not appear dehydrated or malnourished. Ethanol-fed dams had an average blood ethanol level of 163 mg/dl in the morning. On the 22nd day of gestation, animals were euthanized by cervical dislocation. Fetuses were delivered surgically and weighed. They were then decapitated, and blood samples and liver and brain tissues were collected. Fetal brain and liver were rapidly frozen in liquid N_2 and stored at −70°C. Fetal blood and liver IGF data has already been described (14).

Measurement of IGF-I and IGF-II. Frozen fetal brains were weighed individually, powdered with a chilled steel mortar and pestle and pooled for analyses. Using the method of D'Ercole and Underwood (15), IGFs were extracted from 0.1–0.3 g of fetal brain, and IGF-binding protein was separated by gel filtration (16) on 0.9 × 30–cm Sephadex G-75 columns eluted with 1 *M* acetic acid in 0.15 *M* NaCl. IGF-I was measured by nonequilibrium radioimmunoassay (17) using antiserum UB3-189 (obtained from Drs. L. E. Underwood and J. J. Van Wyk through the National Hormone and Pituitary Program) which cross-reacts approximately 0.2% with rat IGF-II and minimally with 10^{-5} *M* insulin. Recombinant human IGF-I (Amgen, Thousand Oaks, CA) was used as standard (0.003–0.8 ng). The sensitivity of this assay was 3 pg or less, and the intra- and interassay coefficients of variation were 5.0% and 7.8%, respectively. IGF-II was measured with a monoclonal antibody against rat IGF-II (Amano, Troy, VA) using rat IGF-II as standards (0.006–1.6 ng, Amano) (18). This assay was sensitive to 6 pg rat IGF-II or less, and the intra- and interassay coefficients of variation were 6.6% and 10.3%, respectively.

Northern Blot Analyses of IGF-I and IGF-II mRNA in Fetal Brain. Total cell RNA was isolated from pooled fetal brains by a single-step procedure using guanidine isothiocyanate lysis followed by phenol-chloroform extraction (19). Twenty micrograms of RNA was separated on 1% agarose gels containing 2.2 *M* formaldehyde and transferred to Gene Screen Plus nylon membranes (NEN Research Products, Boston, MA) by blotting. The quality and integrity of the RNA preparations and equal loading in different lanes were checked by adding ethidium bromide (0.4 μg/ml) to the samples prior to electrophoresis followed by visualization under ultraviolet (UV) light (20). Full-length cDNA inserts specific to rat IGF-I (21) (0.70 kb *Eco*RI fragments of a pro-IGF-I clone) or IGF-II (22) (0.71 kb *Pst*I fragment of a pro-IGF-II clone) were labeled with [^{32}P] by nick-translation and hybridized (3×10^6 cpm/ml) with the RNA on the blots in the presence of 10% dextran sulfate and 50% formamide for 20 hr at 42°C (23). The membranes were washed under highly stringent conditions (15 m*M* sodium chloride–15 m*M* sodium citrate at 65°C) and subjected to autoradiography using Kodak XAR-5 film and intensifying screens at −70°C. The blots were stripped and rehybridized with a [^{32}P]-labeled chicken actin cDNA probe, which was used as a control. Autoradiograms were scanned with a Bio-Rad 620 video densitometer to quantitate mRNA levels. Molecular sizes were calculated using an RNA ladder (9–0.3 kb, GIBCO BRL, Gaithersburg, MD).

Analysis of IGF-Binding Proteins. Fetal brain IGFBPs were analyzed by the slot blot method. IGFBP fractions resolved after acid-gel chromatography were lyophilized and dissolved in Tris-buffered saline (TBS), pH 8.2. The final sample volumes were adjusted to correspond to dilutions of the original brain weight used for the acid-gel filtration. The binding pro-

teins were immobilized on nylon membranes at three different concentrations using a slot blot module and vacuum filtration, and were subjected to ligand blot analysis (24). Briefly, the membranes were treated sequentially with 3% NP-40, 2% nonfat dry milk, and 0.1% Tween-20 followed by incubation with [^{125}I]–IGF-I (0.05 μCi/ml) at 4°C for 15 hr. After extensive washing with TBS/0.1% Tween-20, the blots were subjected to autoradiography and quantitated by densitometric analysis. This procedure allowed quantitative analysis and autoradiography of multiple samples simultaneously, and thus permitted the expression of results from all the treatment groups in units of densitometric analysis per gram tissue weight, rather than relative to the AF control group.

Statistical Methods. All results are expressed as mean ± SEM. Statistical significance was determined using one-way analysis of variance followed by Duncan's multiple range test. The Pearson product-moment correlation coefficient was used to assess relationships between various parameters.

Results

Figure 1 shows mean ± SEM values of fetal body and brain weights of the AF, PF, and EF groups. Ethanol significantly reduced fetal body weight by 17% and 15% compared with AF and PF animals, respectively (4.6 ± 0.1 vs 5.5 ± 0.1 and 5.3 ± 0.1 g; $P < 0.01$; $n = 10$). Brain weight was reduced in EF pups, the actual values being 188 ± 3 vs 211 ± 2 and 203 ± 3 mg for EF, AF, and PF offspring, respectively ($P < 0.01$ or less). The brain to body weight ratio was higher in the EF group than either control group ($P < 0.05$), indicating a relatively greater decrease in body weight than brain weight. The offspring of PF control dams did not show significant change in body or brain weight compared to the AF group.

Ethanol-fed dams consumed less food than AF animals (93 ± 3 vs 148 ± 1 kcal/day; $P < 0.01$) and had lower weight gain during pregnancy (140 ± 17 vs 226 ± 12 g; $P < 0.01$). The change in body weight of PF rats was also less than that of AF control rats (149 ± 14 vs 226 ± 12 g; $P < 0.01$). In contrast, EF and PF dams gained body weight to nearly an equal extent during pregnancy. This suggests that reduced maternal weight gain by EF dams during pregnancy resulted from lower caloric intake rather than from ethanol ingestion.

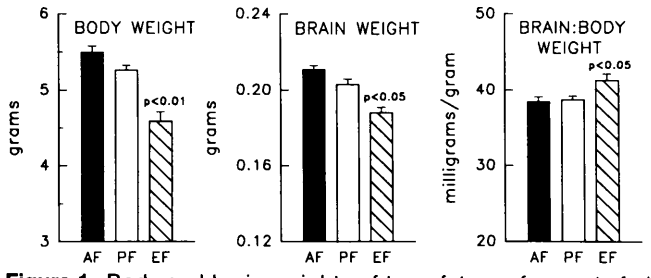

Figure 1. Body and brain weights of term fetuses from rats fed ethanol-containing liquid diet or control diet during pregnancy. AF, *ad libitum* fed control group; PF, pair-fed control group; EF, ethanol-fed group. Results are shown as mean ± SEM; $n = 10$.

Mean ± SEM values of fetal brain IGF-I and IGF-II are shown in Figure 2. Ethanol reduced IGF-I levels significantly ($P < 0.01$) compared with the corresponding PF and AF values (i.e., 2.4 ± 0.2 vs 3.6 ± 0.3 and 4.1 ± 0.4 ng/g for the EF, PF, and AF groups respectively). A positive correlation was observed between brain IGF-I and brain weight ($r = 0.561$, $P < 0.01$). IGF-II levels were not significantly altered by prenatal ethanol exposure.

Figure 3 shows representative northern analyses of IGF-I and IGF-II mRNAs in term fetal brain. RNA samples were loaded in equivalent amounts from each experimental group, as indicated by the ribosomal RNA pattern on the gel used for Northern blotting. No significant change was seen in actin mRNA abundance. The multiple transcripts of both IGF-I and -II are seen routinely and result from a combination of multiple transcription initiation sites, multiple polyadenylation sites, and alternate splicing (25). Higher molecular weight mRNAs for both IGF-I and IGF-II were decreased in the EF and PF groups compared with AF controls. The disappearance of the large size transcripts in EF and PF lanes could not have been due to a differential transfer or unequal loading of RNA, as 28S ribosomal RNA (4.72 kb) was present in equivalent amounts on the blot after methylene blue staining. These species were decreased to a greater extent in the EF group, which also showed significant decreases in the lower-molecular weight species. Because the multiple species encode the same mature peptides, these were pooled for densitometric analysis. Thus, the combined peaks of IGF-I mRNA in the fetal brain of the EF group were decreased by 59% compared with AF controls and by 28% compared with PF controls in 3 experiments. IGF-II mRNA levels in the EF group showed decreases of 50% and 38% compared with AF and PF controls, respectively. In the PF group, fetal brain IGF-I and IGF-II mRNA levels were decreased compared with the AF group by 28% and 21%, respectively, suggesting the effect of undernutrition on IGF gene expression, as has been shown by other investigators (8–10).

Figure 4 shows representative slot blot analyses of IGFBPs in the fetal brain and serum of the offspring of EF, PF, and AF rats. Although the analysis suggested relatively less IGFBP levels in the EF group than control groups, the values were not significantly different, as shown in Figure 2. In contrast, serum IGFBP levels were increased in EF compared to AF and PF rats with an inverse relationship with fetal body weight as described previously (14).

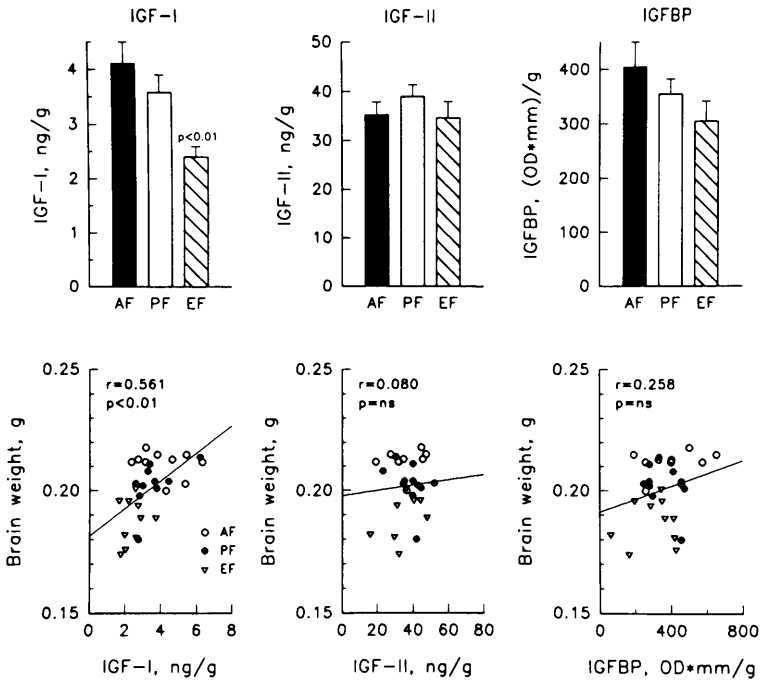

Figure 2. Brain IGF-I, IGF-II, and IGFBP levels in term fetuses of rats fed ethanol liquid diet or control diet during pregnancy. AF, *ad libitum* fed control group; PF, pair-fed control group; EF, ethanol-fed group. Results are shown as mean ± SEM; $n = 10$. r, the Pearson product-moment coefficient. The abundance of IGFBP was determined by densitometric scanning of slot blot autoradiograms. OD*mm, the unit of densitometric quantitation of the peaks.

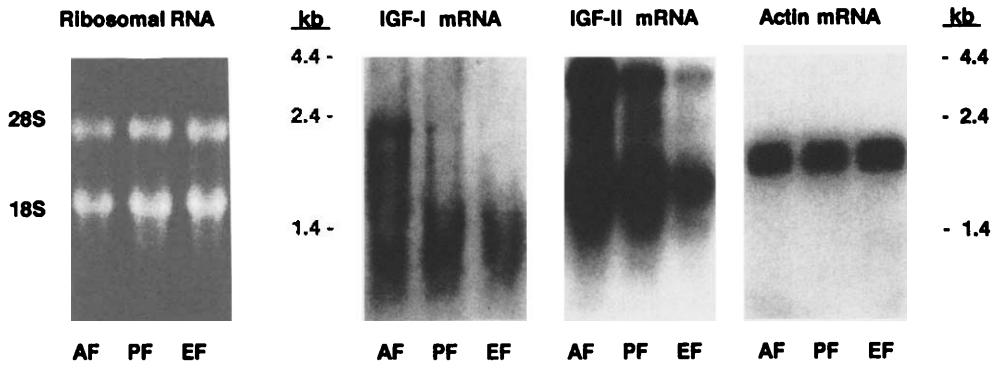

Figure 3. IGF-I and IGF-II mRNA expression in fetal brain. Twenty micrograms of total brain RNA from term fetuses from dams fed ethanol (EF), pair-fed control diet (PF), and fed control diet *ad libitum* (AF) were subjected to Northern blot analysis. The ribosomal RNA pattern, visualized by staining the gel used for northern blotting with ethidium bromide, is shown in the left panel. Hybridization with β-actin cDNA, used as a control, is shown in the right panel.

Discussion

Both IGF-I and IGF-II are present in brain and affect its metabolic and growth processes (4–7). For example, IGFs promote brain cell proliferation, oligodendrocyte differentiation, DNA, RNA and protein synthesis, neurite outgrowth, and synaptogenesis, and also support the survival of glial and neuronal cells in culture. IGF-I administration increases brain protein synthesis and brain growth. In previous reports, we and others have shown that serum IGF-I levels are reduced in offspring of dams given ethanol during pregnancy (12, 14). Also, fetal liver IGF-I levels, as well as the steady-state level of hepatic IGF-I mRNA, are reduced (14). The present study shows that prenatal ethanol exposure decreases fetal brain weight, IGF-I, and steady-state IGF-I mRNA levels. Furthermore, fetal brain weight correlated with brain IGF-I levels. A recent report showed a slight, but nonsignificant decrease in brain IGF-I mRNA level at 10 days postnatal, but not at term in rats exposed to ethanol during gestation (26). A difference in the experimental protocol may be a factor, as ethanol was substituted in lieu of carbohydrates by Breese, Decosta, and Sonntag (26), while in our studies, ethanol replaced primarily dietary fats to minimize alterations in glucose homeostasis.

Intrauterine growth retardation in response to maternal fasting is associated with decreases in IGF levels and their gene expression (9, 10, 27). In our study, IGF-I and IGF-II mRNA levels were reduced in rats that consumed less food than in control rats fed diet *ad libitum*. IGF gene expression was decreased further in ethanol-treated animals when compared with animals

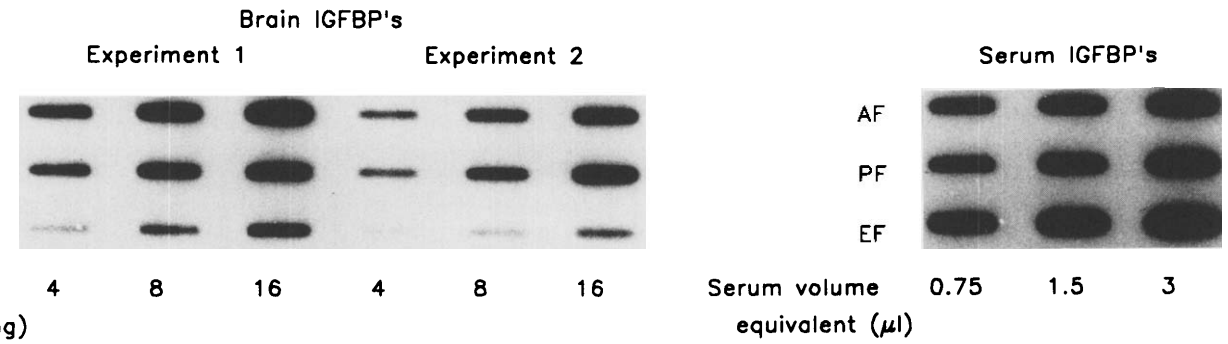

Figure 4. Representative autoradiographic pattern of IGFBP content in fetal brain and serum. Term fetuses from ethanol-fed (EF) and pair-fed (PF) and *ad libitum*–fed (AF) control dams were used as described in the text. Samples of 40, 80, and 160 μl volumes, corresponding to 4, 8, and 16 mg of brain tissue were used for analyses.

fed equicaloric diet. Ethanol inhibits fetal brain uptake of glucose (28), and such a restriction of energy substrates may influence IGF mRNA levels. Other studies have shown that ethanol can inhibit or enhance specific mRNA levels in brain cells (13, 24, 29, 30). Zoeller and Fletcher (31) reported that even a single dose of ethanol decreases c-*jun* mRNA with a concomitant increase in c-*fos* mRNA in the hypothalamus and hippocampus. It is possible that ethanol reduced steady-state IGF mRNA levels by decreasing gene transcription or by destabilization of mRNA during post-transcriptional processes, as it acts on signal transduction systems which alter intracellular cyclic AMP and calcium levels (32, 33). Recently, it has been suggested that prenatal ethanol exposure affects IGF-I mRNA maturation due to delay in CNS development (26).

The observed decrease in fetal brain IGF-I level may be the net result of diminished gene expression and effects on translation, which is known to be inhibited by ethanol (34). Finally, it should be noted that circulating IGFs cross the blood-brain barrier through a receptor-mediated transcytosis mechanism (35). It is possible that the decrease in fetal circulating IGF-I associated with prenatal ethanol exposure may contribute to reduced brain IGF-I levels. Fetal brain IGF-II levels were slightly, but not significantly, decreased. IGF-II gene expression in fetal brain exceeds that of IGF-I, and IGF-II level is higher than that of IGF-I (6). It is possible that the effect of ethanol was not of sufficient magnitude to alter the IGF-II level.

In plasma and extracellular fluids, IGFs are bound to a family of IGFBPs, which provide a reservoir of appropriate concentration of the growth factors for receptor binding (4). Six species of IGFBPs, structurally related to each other, have been identified. These proteins are synthesized in concert with local IGF-I or IGF-II synthesis to modulate IGF actions in an autocrine or paracrine fashion. Bondy *et al.* (5) have shown that IGFBP2 and IGFBP5 are prominently expressed in the brain, and both exhibit peak gene expression which coincides with that of IGF-I. A slight, but insignificant, decrease in IGFBP content was observed in the fetal brain of ethanol-treated rats. This lack of a detectable difference could be due to an effect on only one type of binding protein. Since IGFs increase the recruitment of glial cells expressing IGFBP genes, it is possible that effects on IGFBP could be secondary to the change in IGF-I levels. Another possibility is of inhibition of protein synthesis by ethanol, as has previously been shown in cultured astrocytes (36).

Recent data from the national birth defect monitoring program indicate that the rate of reported cases of FAS among newborns in the United States was 6-fold higher from 1973 to 1993 than in 1973 (37). Abel and Sokol (38) suggested that FAS is perhaps the leading cause of acquired mental retardation in the Western world. Whereas this study and others (12, 14) have shown aberrations in IGF gene expression and IGF-I level in the fetal brain, data from Rubin's laboratory (39) showed that ethanol inhibited the glial cell proliferation response to IGF-I and IGF-I receptor autophosphorylation. Furthermore, ethanol inhibited IGF-I induced tyrosine phosphorylation of insulin receptor substrate-1 and its association with phosphatidylinositol-3 kinase. It is possible that the effects of ethanol on IGF synthesis and actions may play a role in the pathogenesis of brain FAS.

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