Increased Binding of Epidermal Growth Factor to Placental Membranes of Intrauterine Growth Restricted Fetal Rats

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To examine the relationship between nutrient supply and fetal and placental growth, we examined epidermal growth factor (EGF) binding to membranes prepared from placentas of growth-restricted fetal rats. Intrauterine growth retardation was accomplished by unilateral ligation of the uterine artery; fetal rats on the contralateral uterine horn served as controls. Fetal growth restriction was accompanied by decreased placental wt at 19 and 20 days' gestation and significantly decreased placental glycogen content at 20 and 21 days, 30% and 15%, respectively. Placental DNA content and protein/DNA ratios were similar in the growth-restricted and control groups. Specific binding of ^{125}I -EGF was increased in growth-restricted placentas at 19 and 20 days' gestation by 32% and 16%, respectively. ^{125}I -EGF binding at 20–21 days increased linearly with the extent of placental growth restriction. Competitive binding experiments yielded linear Scatchard plots with an increased receptor density in growth-restricted placenta at 19 and 20 days' gestation, 74% and 60%, respectively. Binding affinities for the EGF receptor were similar in the two groups. The increased binding of EGF to membranes prepared from growth-restricted fetal rats may serve to enhance nutrient uptake under conditions of decreased uterine blood flow. (*Pediatr Res* 25: 214–218, 1989)

Abbreviations

EGF, epidermal growth factor
TGF α , transforming growth factor
IUGR, intrauterine growth retarded

Fetal growth and maturation is determined primarily by the fetal genome but can be influenced by a number of important genetic, environmental, and maternal factors. These include a variety of congenital malformations and chromosomal disorders, environmental toxins such as cigarette smoke and alcohol, and maternal medical conditions which can restrict nutrient supply to the fetus. Fetuses with decreased uteroplacental blood flow may exhibit to varying degrees, depending on the severity of substrate and oxygen restrictions, low birth wt with sparing of brain growth, polycythemia, hypoglycemia secondary to defective glucose homeostasis, and accelerated lung maturation. The biologic mechanisms underlying the alterations in growth seen with decreased nutrient supply are poorly understood. In previous work (1–4), we reported that placentomegaly and maturational delay in fetuses of diabetics was associated with a striking decrease in the binding of epidermal growth factor to membranes prepared from the diabetic placentas. In light of these observations and because of the role of EGF in cellular proliferation and

organ differentiation in numerous cell types and animal species (5–7), we examined EGF binding to membranes prepared from placentas of fetal rats with growth restriction secondary to uterine artery ligation. In these studies, we raise the possibility that EGF binding to placental membranes is regulated by substrate flux and is associated with maturational changes in diabetic and IUGR fetuses.

MATERIALS AND METHODS

Animal model. Pregnant adult female Sprague-Dawley rats were obtained from Holtzman Lab Animals (Madison, WI) at 7–9 days' gestation (the morning that sperm were detected was designated as day 0 of pregnancy). Females were maintained on a 12/12 h light/dark cycle at 24°C, and given food and tap water *ad libitum* for 7–9 days before animal surgery. On days 16, 17, or 18 of gestation, unilateral uterine artery ligation was performed by a modification of the method of Wigglesworth (8) under Metofane (Pitman-Moore, Washington Crossing, NJ) inhalation anesthesia as follows. A midline incision of the lower abdomen was made, and the uterine horns were exteriorized and inspected. A single 3.0 silk ligature was placed securely around the main branch of the uterine artery close to the cervix. Only horns containing six or more fetuses were used for operation; the contralateral horn in each animal served as a control. The uterine horns were then replaced within the abdominal cavity; the peritoneum was closed with continuous sutures and the skin with wound clips.

Animals were killed by decapitation 3 days after surgery at 19, 20, or 21 days' gestation. Surviving fetuses and placentas from both uterine horns were weighed and immediately placed on ice for further dissection and analysis. Animal studies were carried out after appropriate institutional review.

Preparation of crude cell membrane fraction. Placentas were removed and weighed immediately after animals were killed. The membrane fraction was prepared by a modification of the method of Morishige *et al.* (4, 9). Tissue was homogenized in a polytron homogenizer in 10 vol of 0.3 M sucrose in 25 mM Tris-HCl, pH 7.6, containing 10 mM MgCl₂. The resulting homogenate was centrifuged at 800 $\times g$ for 10 min, and the supernatant was then centrifuged at 20,000 $\times g$ for 20 min. The pellet was washed with 20 vol of 25 mM Tris-HCl, pH 7.6, containing 10 mM MgCl₂ and underwent centrifugation at 20,000 $\times g$ for 10 min. This wash was repeated twice. The final pellet was resuspended in 25 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 0.5% BSA at a dilution to yield approximately 3 mg protein/ml. All procedures were performed on ice or at 4°C. This fraction was then used in the binding experiments.

^{125}I -EGF binding. Binding assays were carried out in a total vol of 0.25 ml in 1.5 ml polypropylene tubes. Each tube contained 300 μg placental membrane protein, 30,000–40,000 dpm ^{125}I -EGF (0.10–0.20 ng), sp act of 150–200 $\mu\text{Ci}/\mu\text{g}$ (New England

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Nuclear, Boston, MA) and increasing amounts of unlabeled receptor grade EGF (Collaborative Research, Bedford, MA) in 25 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 0.5% BSA. The tubes were shaken continuously for 90 min at room temperature, at which point the reaction was terminated by the addition of 0.75 ml 50 mM Tris-HCl, pH 7.6. The tubes were centrifuged for 10 min at $10,000 \times g$ in a Beckman microcentrifuge (Beckman Instruments, Palo Alto, CA). The supernatant containing unbound EGF was removed and the pellet counted for 10 min in a Packard 5110 γ -counter (Hewlett-Packard Co., Palo Alto, CA). Nonspecific binding was determined by the amount of ¹²⁵I-EGF bound in the presence of 1000 ng unlabeled EGF and was subtracted from all values to determine the amount of EGF specifically bound. EGF receptor number and affinity were determined by Scatchard analysis of the competitive binding assay data (10).

Degradation of ¹²⁵I-EGF. To assess possible differences in degradation of ¹²⁵I-EGF tracer, specific binding was determined for membranes prepared from control and IUGR rat placentas and human placenta. The supernatant fractions remaining after the initial binding assays were each reincubated with human placental membranes, and specific binding was then compared to the initial binding for the human placental membranes.

Rebinding experiment. Magnesium chloride was used to dissociate EGF bound to receptor in the membrane preparations as described by Kelly *et al.* (11). Specific binding assays were performed as above for both membrane preparations. Immediately after counting, the pellets were resuspended in 0.5 ml of 1 M MgCl₂ in binding assay buffer, vortexed, and incubated for 5 min. The reaction was terminated by the addition of 3.5 ml of the binding assay buffer. The tubes were centrifuged at $2300 \times g$ for 15 min. After aspirating the supernatant, the pellet was counted and resuspended, and the binding assay was repeated.

Radioreceptor assay for EGF. The amount of EGF-like activity in amniotic fluid and fetal serum was measured by radioreceptor assay. Human placental membranes were isolated according to a modification of the method of Hock and Hollenberg (4, 12) and suspended at a concentration of 5- μ g membrane protein per tube. The assay mixture contained 30,000 to 40,000 dpm ¹²⁵I-EGF, varying amounts of cold receptor grade EGF (0–1000 ng/tube), or test sample in a final vol of 300 μ l. The binding mixture was incubated in Dulbecco's PBS, 250 mM HEPES, 0.1% BSA, pH 7.6, at room temperature with continuous shaking for 60 min. The reaction was terminated by the addition of 1 ml of ice-cold incubation buffer and filtered through Whatman GF/C filters using the Millipore 3025 sampling manifold (Millipore Continental Water Systems, Bedford, MA) to separate bound from unbound ligand. The assay tube was washed once with 1 ml ice-cold buffer, and the filter was rinsed three additional times with ice-cold buffer. The filter was then counted in a Packard 5110 γ -counter. A displacement curve of bound ¹²⁵I-EGF versus log EGF concentration was constructed, and the EGF-like activity present in the test sample was estimated by the amount of ¹²⁵I-EGF displaced.

Glycogen analysis. Samples of placentas and liver were weighed rapidly, flash frozen in liquid nitrogen, and stored at -70°C until analysis. Glycogen content was measured as described previously (13).

Protein and DNA analysis. Total protein was measured by the method of Lowry *et al.* (14), using BSA as the standard. DNA was assayed by the method of Burton (15), using deoxyribose as the standard.

Statistical analysis. Student's *t* test was used to compare results between control and growth-restricted groups. Data is expressed as mean $\pm$ SE.

RESULTS

Growth restriction. Unilateral uterine artery ligation resulted in variable survival on the ipsilateral uterine horn; a surviving

fetus, for the purpose of these studies, was defined as having obvious spontaneous body movements within the amniotic sac and no evidence of resorption. The range of somatic growth restriction observed in individual fetal rats was from 0–40% less than the mean body wt of the fetuses on the contralateral uterine horn.

We confined our studies to those fetal rats with substantial growth restriction, defined for these studies as a $\geq 20\%$ reduction in body wt as compared to controls from the contralateral horn. Body wt comparisons at 19, 20, and 21 days' gestation are shown in Figure 1. The liver glycogen content was decreased by 39% in IUGR fetal rats, an observation consistent with previous studies using the Wigglesworth system of surgically induced IUGR (8).

Standardization of ¹²⁵I-EGF binding assay. Figure 2 shows the binding of ¹²⁵I-EGF as a function of increasing membrane protein. The specific binding was linear over the range of 100–700 μ g protein. Total and specific binding of ¹²⁵I-EGF also increased linearly from 30,000 to 300,000 dpm of iodinated EGF.

Figure 3 shows the time course of binding to membranes prepared from placentas of control and IUGR rats. Specific binding was greater in the IUGR placental membranes than in controls over the entire 2-h time course. The ratio of binding in

Fig. 1. Comparison of body wt of control and IUGR fetal rats at 19, 20, and 21 days' gestation. Data represented as means $\pm$ SE.

Fig. 2. Standardization of ¹²⁵I-EGF binding to placental membrane: binding versus membrane protein.

IUGR rats over controls remained consistent, making it unlikely that the increased binding of EGF to placental membranes from IUGR rats was due to different rates of EGF degradation.

Dissociation of the EGF-receptor complex. To determine whether or not the increase in ^{125}I -EGF binding to membranes prepared from IUGR rats was due to a difference in occupancy of the receptor, MgCl_2 was used to dissociate the ligand-receptor complex. Figure 4 shows the results of this experiment. Specific binding before dissociation and after rebinding reveals increases of 28 and 35%, respectively, for placental membranes from IUGR rats. As the ratio of EGF bound before and after dissociation is approximately equal, it would appear that the receptors in both membrane preparations have the same degree of occupancy.

Placental biochemical studies. Placental wt, protein, DNA, and glycogen content at 19, 20, and 21 days' gestation are shown in Table 1. IUGR placentas were decreased in size, as compared to control placentas, at 19, 20, and 21 days' gestation by 8%, 14%, and 13%, respectively. These differences were statistically significant at 19 and 20 days. Placental growth restriction was less dramatic than fetal growth restriction, as demonstrated by the differences in fetal/placental ratios (F/P) with advancing gestational age (Fig. 5). The DNA concentrations in controls were not significantly different than those found in the IUGR placentas. Although the protein concentration at 21 days' gestation was slightly increased in IUGR placentas, the protein/DNA ratios were similar at the 3 gestational days which were studied. Placental glycogen content was significantly decreased in IUGR placentas at 20–21 days' gestation by 30% and 15%, respectively.

Comparative binding of ^{125}I -EGF in control and IUGR placenta. The specific binding in IUGR placentas at 19, 20, and 21 days is increased by 32%, 16%, and 7%, respectively, with the differences being statistically significant at 19 and 20 days' gestation. Specific binding *versus* placental growth (measured by wet wt) in IUGR fetuses at 20–21 days' gestation, as shown in Figure 6, suggests a linear relationship between the extent of growth restriction in individual placentas and the binding of ^{125}I -EGF. This relationship was not observed at 19 days' gestation.

Competitive binding experiments provided data that yielded linear Scatchard plots on days 19 and 20 of gestation in both control and IUGR placentas. Figure 7 shows a representative Scatchard plot of day 19 placenta membranes from control and IUGR rats. Linear regression analysis was used to estimate the receptor density and binding affinity (K_d) of control and IUGR placentas for ^{125}I -EGF on days 19 and 20 (Table 2). There was an apparent increase in receptor number in IUGR placental membranes on both days. Receptor density was increased in

Fig. 3. Time course of ^{125}I -EGF binding to placental membrane from control and IUGR rats: binding *versus* time of incubation.

Fig. 4. MgCl_2 removal of EGF bound to placental membranes from control and IUGR rats.

Table 1. Placental characteristics*

	Placental wt (mg wet wt)	DNA (mg/g wet wt)	Protein (mg/g wet wt)	Glycogen ($\mu\text{g}/\text{mg}$ protein)
Day 19				
Control	563 $\pm$ 9 (50)	1.24 $\pm$ 0.07 (4)	99 $\pm$ 9 (7)	25.4 $\pm$ 5.5 (7)
IUGR	519 $\pm$ 14 (39)	1.39 $\pm$ 0.03 (4)	99 $\pm$ 9 (7)	24.6 $\pm$ 5.5 (7)
Day 20				
Control	622 $\pm$ 14 (49)	1.27 $\pm$ 0.12 (8)	105 $\pm$ 5 (18)	14.3 $\pm$ 1.0 (12)
IUGR	535 $\pm$ 16 (42)	1.21 $\pm$ 0.12 (8)	108 $\pm$ 3 (16)	10.0 $\pm$ 0.5 (11)
Day 21				
Control	618 $\pm$ 27 (29)	1.35 $\pm$ 0.03 (8)	123 $\pm$ 4 (8)	13.0 $\pm$ 0.5 (8)
IUGR	540 $\pm$ 34 (30)	1.43 $\pm$ 0.02 (8)	134 $\pm$ 4 (8)	11.1 $\pm$ 0.3 (8)

* Values are $\pm$ SEM with n in parentheses.

Fig. 5. Fetal/placental wt ratios of control and IUGR fetal rats at 19, 20, and 21 days' gestation. Data represented as means $\pm$ SE.

IUGR placentas at 19 and 20 days by 74% and 60%, respectively. The EGF receptors in control and IUGR placentas have similar binding affinities.

Degradation of ^{125}I -EGF. Supernatants from control and

IUGR membrane preparations were each reincubated with human placental membranes. The specific binding was essentially equal. If the difference in binding seen in the membranes from control and IUGR rats were due to a higher rate of tracer degradation from the control membrane preparation, one would expect to see a similar decrease in binding in the supernatant fraction from the control tissue.

Radioreceptor assay. Results from radioreceptor assays for EGF-like activity are shown in Table 3. Levels of activity in fetal serum and amniotic fluid at 20 days' gestation were not significantly different between the control and IUGR groups.

DISCUSSION

We reported previously that membranes prepared from placentas of rats with streptozotocin-induced diabetes showed a striking decrease in the binding of EGF when compared with binding to control placental membranes (4). On days 21–22, this decrease in binding related primarily to the failure of a second class of lower-affinity receptors to appear in the diabetic placenta. We speculated that the excessive substrate supply to the diabetic fetuses resulted in sustained cell division and a concomitant delay in maturation of the diabetic placentas and that these alterations in placental growth and maturation may be modulated by the changes in EGF binding.

To examine further the relationships between substrate supply, EGF binding and placental maturation, we prepared placental membranes from fetal rats with growth restriction induced by uterine artery ligation (8). Fetuses were characteristically reduced

in wt by at least 20%. Nitzan *et al.* (16) observed a large reduction in the placental transfer of glucose and amino acids to fetal rats with surgically induced growth restriction, but oxygen transfer is also likely diminished. The decreased availability of glucose in IUGR fetuses is associated with decreased accumulation of hepatic glycogen near the end of gestation, which we also observed.

The restriction of overall fetal growth was accompanied by decreased placental wt. Cell division within the rat placenta, as measured by DNA content, has essentially ceased by 16–17 days' gestation (1), which is before the time of uterine artery ligation. It is, therefore, not surprising that placental DNA concentrations of the IUGR fetuses were not significantly different from controls between 19 and 21 days' gestation. Placental growth during the last 5–6 days of the rat pregnancy represents hypertrophy, *i.e.* is associated with increases in cell protein and RNA, as shown by Winick and Noble (17). Placental protein in IUGR placentas in our studies was not significantly different than in controls at 19 and 20 days' and actually slightly increased at 21 days' gestation. Moreover, protein/DNA ratios were similar; therefore, the observed decrease in placental growth was unlikely to have been the result of protein malnutrition. Although the placental weights showed significant differences on days 19 and 20, the protein/DNA ratios did not reach statistical significance on any of the days studied. Placental glycogen content, which has previously been shown to decrease in the rat pregnancy from 16 days onward (1), was further decreased in IUGR placentas at 20 and 21 days'

Table 2. Receptor density and binding affinity*

Day	Group	Receptor density (pmol/mg protein)	Binding affinity (Kd) (nM)
19	Control	85.1 ± 6.0 (n = 5)	0.49 ± 0.05 (n = 5)
	IUGR	148 ± 28.3 (n = 4)	0.58 ± 0.07 (n = 4)
		p < 0.05	NS†
20	Control	104.9 ± 13.2 (n = 4)	0.62 ± 0.08 (n = 4)
	IUGR	168.4 ± 9.6 (n = 4)	0.59 ± 0.03 (n = 4)
		p < 0.01	NS

* n represents the total number of binding experiments, each of which involved the measurement of competitive binding of ¹²⁵I-EGF in membrane prepared from three to five placentas from the same pregnant animal.

† NS, not significant.

Table 3. Levels of EGF-like material in amniotic fluid and fetal serum*

Group	Serum (ng/ml)	Amniotic fluid (ng/ml)
Control	13.2 ± 0.9 (n = 5)	0.75 ± 0.12 (n = 6)
Growth-Restricted	12.0 ± 0.6 (n = 4)	0.69 ± 0.14 (n = 4)
	NS†	NS

* Radioreceptor assay was carried out at 20 days' gestation as described in the text.

† NS, not significant.

Fig. 6. Specific binding of ¹²⁵I-EGF versus placental wt in IUGR placentas of 20–21 days' gestation.

Fig. 7. Scatchard analysis of binding of ¹²⁵I-EGF to placental membranes from control and IUGR fetal rats at day 19 of gestation.

gestation. There may also have been quantitative differences in water content or other cellular constituents (*e.g.* cell membrane phospholipids) not measured in these studies that were responsible, in part, for the diminished tissue wt in IUGR placentas.

Diminished placental glycogen content has been observed in many species toward the end of normal gestation (18, 19) and is thought to occur secondary to the increased utilization of substrate for biosynthetic pathways by the fetal-placental unit (20). The decrease in glycogen content of IUGR placentas at 20–21 days' gestation may also be secondary to oxygen- and nutrient-deprivation.

The increased specific binding of EGF by IUGR placentas and the apparent relationship between the extent of placental growth restriction and EGF binding suggests that the EGF receptor may play a role in a nutrient-deprived environment. EGF stimulates the uptake of glucose, amino acids, and uridine (21–23) and is a mitogen (24, 25) in various *in vitro* systems. EGF receptor number increases toward the end of gestation in mice (26), rats (4), and humans (27), suggesting a relationship between maturation and EGF binding. The increased binding of EGF to membranes from IUGR fetuses may serve to enhance nutrient uptake under conditions of decreased uterine blood flow.

The changes in binding of EGF at days 19 and 20 of gestation in the IUGR rat placentas occurred secondary to an apparent increase in receptor number. The binding affinity of the receptor was not significantly different between IUGR and control placentas. EGF itself can cause synthesis of the EGF receptor, so-called up-regulation (28). Glucocorticosteroids can enhance EGF binding (29). Our studies have not, as yet, included the measurement of glucocorticosteroid levels or binding in control and IUGR fetal rats. Stress-associated increases in glucocorticosteroid levels in the IUGR fetus would not be surprising. The measurement of EGF-like activity as performed in the radioreceptor assay revealed no significant differences in levels between control and IUGR animals in either serum or amniotic fluid. Therefore, up-regulation by EGF would appear to be a less likely mechanism for the increased specific binding and apparent receptor density in IUGR placentas. It is important, however, to point out that the growth factor TGF α (30) has been demonstrated to bind with specificity to the EGF receptor, so measurements of EGF activity levels, as determined by radioreceptor assay, should be interpreted as EGF-like activity.

Finally, one could speculate that increased mRNA for the EGF receptor, or increased transcription of that message, results in an increase in synthesis of the receptor in IUGR placentas. EGF has been associated with increases in glucose and amino acid uptake in isolated cells. The greater receptor density as a response to nutrient deprivation suggests the possibility of an EGF-mediated increase in nutrient transport. Studies to quantify specific mRNA for the EGF receptor in control and IUGR placentas are currently in progress.

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