Influence of Maternal Diabetes in Rats on Hemoglobin Synthesis and Uridine Uptake by Fetal Liver Cells

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SUMMARY

The effect of streptozocin (STZ)-induced maternal diabetes in rats on fetal erythropoiesis was studied in short-term cultures of fetal liver cells at the time of switch from embryonic to adult hemoglobins. Liver erythroid cell functions were monitored by measuring the incorporation of [^{3}H]-uridine in trichloroacetic acid (TCA)-soluble and -insoluble cell fractions and of [^{3}H]-leucine in hemoglobin chains. Fetal liver cells of diabetic rats showed a higher incorporation of [^{3}H]-uridine compared with controls when the cells were obtained from 14-day-old fetuses. However, there were no significant differences in the uptake of uridine when cells were obtained from 16-day-old fetuses. In parallel cell cultures, incorporation of [^{3}H]-leucine into adult and embryonic globin chains was studied by separation of the globin chains by high-performance liquid chromatography (HPLC). The overall globin chain synthesis was higher in the fetuses of diabetic mothers compared with controls on day 14 of gestation. Erythropoietin had similar effects on the stimulation of globin chains in the two groups of fetuses. However, in the fetuses of diabetic mothers, erythropoietin had a specific stimulatory effect on embryonic-type globins that was significantly higher in the fetuses of diabetic mothers compared with controls. Differences between fetuses of control and diabetic mothers completely disappeared at 16 days of gestation. It is concluded that maternal diabetes has an effect on the cells synthesizing embryonic hemoglobins on day 14 of gestation, but by the time the switch from embryonic to adult-type hemoglobins is complete, these differences are abolished. DIABETES 1985; 34:212–16.

Maternal diabetes is characterized by glucose intolerance and a general disturbance of metabolic homeostasis that is associated with a number of abnormalities of growth and development of the fetus.[1] Fetal hyperinsulinemia[2] has been implicated as the causative factor in the overgrowth of the fetus[3] as well as other abnormalities, such as increased incidence of congenital malformations.[4] In the neonatal period, the infant of the diabetic mother (IDM) also suffers from a number of complications, such as neonatal hypoglycemia[1] and respiratory distress syndrome.[5] In addition, erythrocytosis, normoblastemia, and extramedullary erythropoiesis occur during the perinatal period in IDM, although the mechanisms responsible for these changes remain obscure.[1]

Widness et al.[6] observed increased erythropoiesis and elevated erythropoietin levels in IDM as well as in hyperinsulinemic rhesus monkey fetuses, although they found no correlation between plasma erythropoietin levels and RBC or reticulocyte counts. Their studies suggest that insulin may play a direct role in fetal erythropoiesis in diabetic pregnancy. However, it has also been suggested that the effects of high levels of insulin on erythropoiesis may be secondary to fetal hypoxia, because Carson et al.[7] observed progressive fetal hypoxia in ovine fetus infused with insulin; however, there is no direct confirmation for this hypothesis.

Erythropoiesis in fetal and adult life is regulated by erythropoietin. In addition, a number of hormones exert an effect on this process, including thyroxine, testosterone, and glucocorticoids.[8,9] Although a direct role for insulin is suggested by the studies of Widness et al.,[6] nothing is known about the interactions of this and other hormones with erythropoietin in the regulation of fetal erythropoiesis. The STZ-induced diabetic rat model offers us the opportunity to investigate the etiology of stimulated red cell production in diabetic pregnancy. In this paper, we describe the effect of maternal diabetes on hemoglobin synthesis in the fetus at the time of the switch from embryonic to adult hemoglobin. We have also evaluated the role of experimental diabetes on uridine uptake by fetal liver cell cultures because it is a sensitive parameter of red cell activity in mice[10] and calf fetuses.[11]

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MATERIALS AND METHODS

Induction of diabetes and collection of fetuses. Sprague-Dawley, virgin females (200–225 g) obtained from Charles River Canada (St. Constant, Quebec) were housed overnight with fertile males; the following morning, females with evidence of sperms in vaginal washings were assumed to be pregnant (day 0 of pregnancy) and were randomly assigned to two groups. Streptozocin (STZ, 45 mg/kg, Sigma, St. Louis, Missouri) or saline-citrate buffer was administered intravenously (i.v.) on day 2 of gestation. Urinary glucose levels were monitored with test tape (Eli Lilly and Company, Toronto) and animals that became glycosuric within 48 h were selected for the study. On days 14 and 16 of gestation, females were anesthetized with ether. Blood was collected by cardiac puncture, the entire uterus was removed and placed in sterile Hanks' balanced salt solution (HBSS). Fetal livers from a minimum of three litters were pooled for each group.

Cell cultures. Fetal liver cell suspensions were prepared by mechanical disruption as previously described for calf fetuses.[11] Briefly, pooled liver pieces were forced through a Pasteur pipette several times and then through a 20-gauge needle. The dispersed cells were filtered through 8 layers of cheesecloth and the cell suspension was centrifuged at 700 × *g*. The upper layer of the pellet containing a yellow ring of cells (cell debris and dead hepatocytes) was discarded and the remaining cells were resuspended in a modified Ham's F-12 medium containing bovine albumin and transferrin.[11] This method of cell preparation destroyed parenchymal cells as efficiently as the trypsin method[11,12] because <1% hepatocytes remained in the cell suspensions used for cell cultures and did not survive during culture, as determined by the trypan blue staining techniques. Cells were cultured in the same medium for 20 h in the presence and absence of erythropoietin (step 3; Connaught Laboratories, Toronto) described elsewhere.[11]

Uridine incorporation. Cultured fetal liver cells were incubated for 1 h with 6 μCi [5-^{3}H]-uridine (25–30 Ci/mmol; New England Nuclear Corporation, Boston, Massachusetts). The incubation with [^{3}H]-uridine was stopped by addition of 10 ml of HBSS. The cells were washed twice, mixed with 1 ml ice-cold 0.75 M trichloroacetic acid (TCA) and left at 0°C for 20 min. The cold acid-insoluble material was removed by centrifugation. Aliquots of the acid-soluble pool (containing mostly nucleoside phosphates) and acid-insoluble fraction (containing RNA) were taken for determination of radioactivity as previously described for calf liver cells.[11]

Leucine incorporation. Fetal liver cells were incubated with 20 μCi [^{3}H]-leucine (158 Ci/mmol; New England Nuclear) for 4 h. Cells were washed 5 times with HBSS as indicated above for uridine incorporation studies and the cells were stored frozen at −40°C. Labeled globin chains were separated using reversed-phase high performance liquid chromatography (HPLC) as described elsewhere.[13]

Other determinations and statistics. Plasma glucose levels were measured on a Beckman autoanalyzer based on a glucose-oxidase method.[14] The values for uridine incorporation and leucine incorporation are expressed as mean ± SEM. These experiments were repeated eight times or more with 14-day-old fetuses and five times or more with 16-day-old fetuses. The experiments were always planned as pairs (controls/diabetics, controls/erythropoietin) to use the paired *t*-test as a test for significance.[15] A probability of <0.05 was considered to be statistically significant. Plasma insulin levels were measured as described previously.[16]

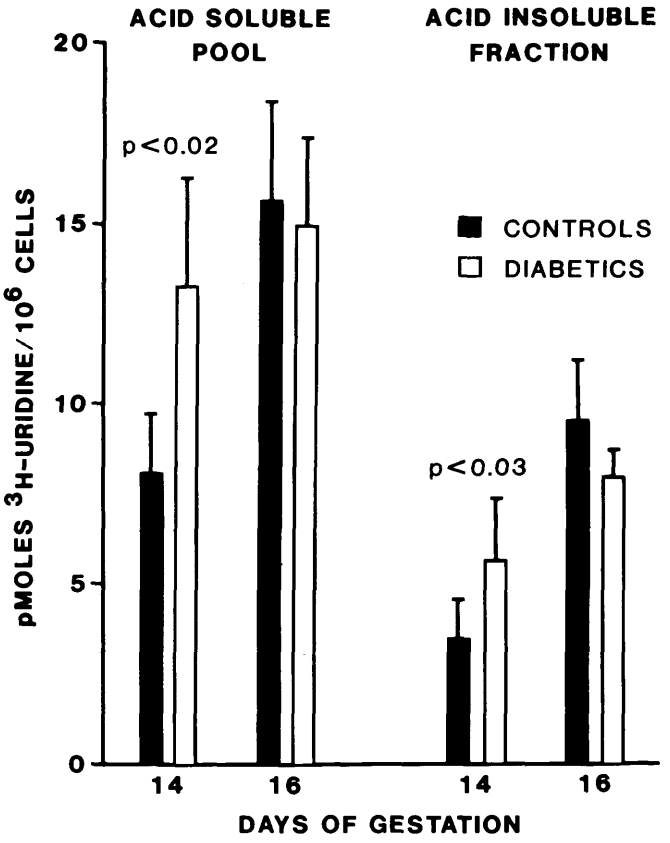

FIGURE 1. Effects of experimental diabetes on [^{3}H]-uridine incorporation into acid-soluble and acid-insoluble cell fractions from fetal liver cell cultures of control (■) and diabetic (□) rats at 14 and 16 days of gestation.

TABLE 1
Maternal plasma glucose and insulin levels

Gestational age	Plasma glucose (mg/dl)		Insulin (ng/ml)	
	Control	STZ-treated	Control	STZ-treated
14 Days	126 ± 7	458 ± 17	3.7 ± 0.8	1.1 ± 0.3
16 Days	96 ± 7	489 ± 13	2.9 ± 0.8	0.8 ± 0.3

STZ, streptozocin. The values are mean ± SEM of 6–10 experiments.

RESULTS AND DISCUSSION

We have previously shown that administration of a 45-mg/kg dose of STZ to pregnant rats produced a diabetic state that resulted in maternal and fetal plasma glucose levels of 20 mM during the last 3 days of gestation.[16] In these rats, the fetuses were hyperinsulinemic but not macrosomic and both maternal and fetal plasma corticosterone levels were reduced, although there was no discernable change in the cytoplasmic glucocorticoid receptors in fetal liver and lung between days 19 and 22 of gestation.[16] Table 1 shows that maternal plasma glucose and insulin levels at days 14 and

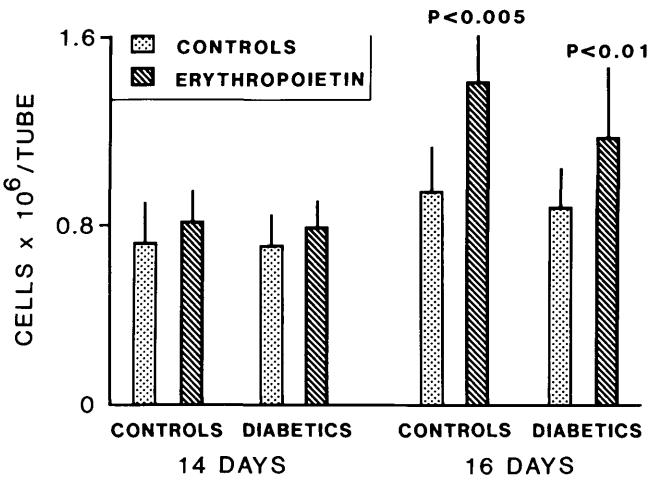

FIGURE 2. Effects of 20-h incubation with 0.2 U/ml erythropoietin on cell numbers of liver cultures of 14- and 16-day-old fetuses. Equal numbers of cells (10^6 cells) were plated for both gestational ages (time 0).

16 of gestation were similar to those found at later gestational ages.[16] For technical reasons, we were unable to measure fetal plasma glucose and insulin levels. However, other investigators have shown that, in the developing rat fetus, the pancreas starts secreting insulin by day 14 of gestation[17] and fetal hyperglycemia was associated with hyperplasia and degranulation of the fetal pancreas at 20 days of gestation,[18] suggesting that, in the moderately diabetic rat, fetal plasma insulin levels may be elevated as early as 14–16 days of gestation.

The rationale for measuring uridine incorporation has been that it is a rapid and a sensitive method for measuring erythropoietic response.[11] Uridine uptake and incorporation into different types of RNA is one of the many pleiotropic effects of erythropoietin. The label present in the acid-soluble fraction corresponds mainly to phosphorylated products of uridine and, in particular, UTP, the immediate precursor for the synthesis of RNA. The acid-insoluble fraction contains all types of RNA. Although uridine incorporation is not a typical erythroid function, it does indicate erythroid activity when tested in a cell culture system devoid of other cell types.

We have studied in detail [^{3}H]-uridine uptake and hemoglobin synthesis at two defined gestational ages, namely 14 and 16 days. The choice of these gestational ages has been made both for practical and theoretical reasons. The period of 14–16 days of gestation in the rat corresponds to the time of the switch from embryonic to adult hemoglobin synthesis.[13] Furthermore, the 14-day-old fetuses can supply a convenient number of cells for the experiments described here. In addition, stimulation of globin chain[13] and heme[19] synthesis by erythropoietin has been studied extensively in fetal liver cells at these gestational ages.

Figure 1 shows the changes observed in uridine uptake both in the acid-soluble and -insoluble cell fractions in liver cells prepared from 14- and 16-day-old fetuses. Uridine uptake in both cell fractions was significantly higher in cultures from fetuses of diabetic mothers at 14 days of gestation but not at 16 days of gestation, suggesting a higher erythropoietic activity at 14 but not at 16 days of gestation. Figure 2 shows that this difference is not due to differential survival of cells from fetuses of diabetic mothers in the in vitro cell culture system, because the number of cells is identical in cultures from fetal livers of control and diabetic mothers at day 14 of gestation. Furthermore, erythropoietin does not significantly increase the number of cells in the cultures at this gestational age. In contrast, erythropoietin caused a significant increase in the number of cells in cultures from 16-day-old fetuses. This difference in cell numbers has been taken into account in all calculations involving the effects of erythropoietin on uridine uptake or globin chain synthesis to be described below.

It is not clear why experimental diabetes should have a differential effect on uridine uptake at 14 days and 16 days of gestation. There are at least two possible explanations for these results. One explanation could be that changes occur within a given cell population at 14 and 16 days of gestation, possibly at the receptor level. The disappearance of the effect of maternal diabetes at 16 days of gestation could be a consequence of downregulation of erythropoietin receptors in cells previously exposed to high levels of erythropoietin at 14 days of gestation and it may serve as a compensatory mechanism in an attempt to restore normal blood cell function after acute stimulation by erythropoietin. Indeed, as shown in Figure 3, cells obtained from fetuses of diabetic mothers are less responsive to erythropoietin than are cells from normal fetuses. Although the amplitude of this difference altered from one experiment to another, multiple linear regression analysis indicated that the difference between the two groups was significant. However, this change may be too small to account for the elimination of the effect of diabetes at 16 days and, at this stage, may be hypothetical because there is only indirect evidence for the existence of erythropoietin receptors in mammalian cells.[20,21] Another

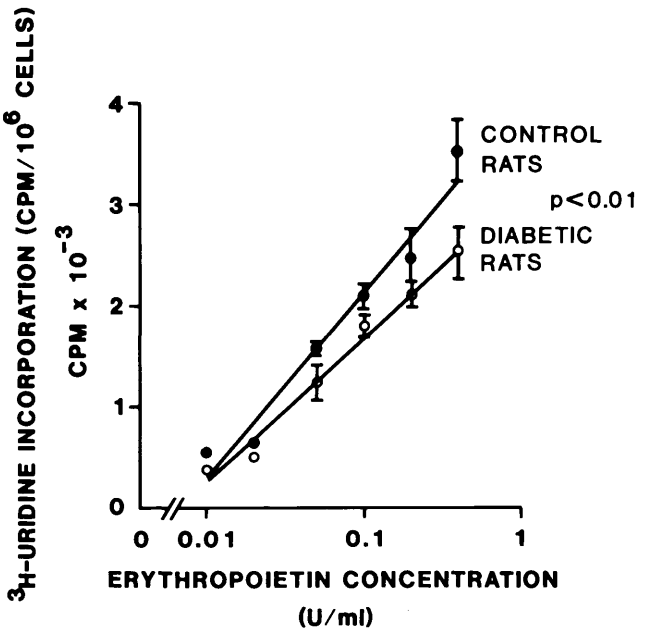

FIGURE 3. Effect of increasing concentrations of erythropoietin on the incorporation of [^{3}H]-uridine into acid-insoluble cell fractions from fetal *liver cell cultures of control (●) and diabetic rats (○) at day 14 of gestation.*

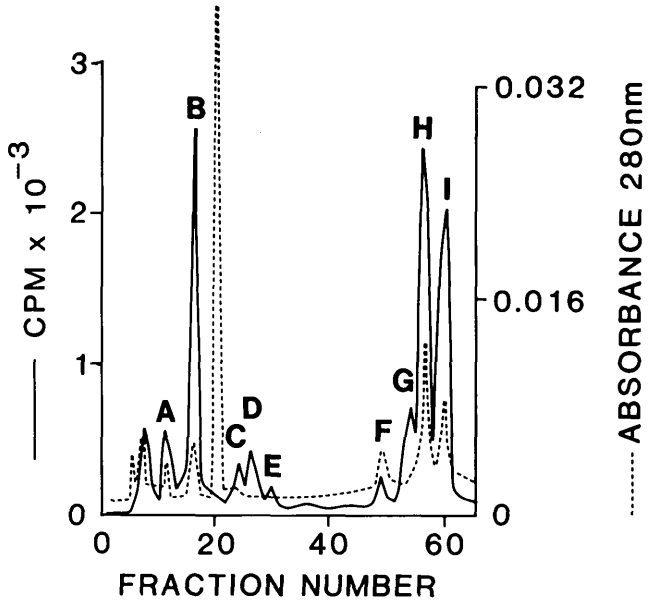

FIGURE 4. HPLC separation of globin chains synthesized by liver cell cultures of 14-day-old control fetuses. Continuous line represents cpm, while dotted line represents absorbance at 280 nm. The absorbance peak at fraction 20 without leucine incorporation corresponds to heme.[13]

possible explanation for the above observations could be that there are two cell populations, one that synthesizes embryonic hemoglobin and the second, which synthesizes adult hemoglobins. It is possible that, at 14 days of gestation, only the cells that synthesize mainly embryonic hemoglobins[13] are sensitive to the effects of experimental diabetes. It is likely that these cells are then replaced at day 16 by new, adult-type cells that synthesize adult hemoglobin, and are less sensitive to the hormonal changes occurring during experimental diabetes. This second interpretation can be tested by measuring embryonic and adult hemoglobin synthesis in cells obtained from fetuses at days 14 and 16 of gestation.

Figure 4 shows the HPLC separation of globin chains synthesized by liver cell cultures obtained from 14-day-old fetuses. There are at least nine different globin chains. We have shown that the first fractions A and B are alpha chains.[13] They are present in both embryonic and adult hemoglobins and are synthesized in cells from 14- and 16-day-old fetuses. The second group of chains (C, D, and E) are beta chains typical of adult hemoglobins, and are synthesized mainly in cells from 16-day-old fetuses. The last chains to be eluted from the columns are embryonic chains, synthesized in liver cells from 14-day-old fetuses.[13] One of the advantages of the HPLC system used is that the embryonic chains are well separated from the adult beta chains. It is then easy to follow their synthesis during gestation. Figure 5 shows that globin chain synthesis is higher in fetuses of diabetic mothers at day 14, thus confirming the higher erythropoietic activity in experimental diabetes. Note that the effect of diabetes is specific for alpha- ($P < 0.04$) and embryonic-type chains ($P < 0.05$) and does not have any significant effect on globin chain synthesis in cells of fetuses at day 16 of gestation. The difference in beta chains was not significant at the 0.05 level on day 14. This is probably due to a methodologic problem, the levels of beta chain synthesis being too low and, therefore, subject to larger experimental variation than the determination of alpha or embryonic chain synthesis. These results are in keeping with the second interpretation of the results indicated above, namely the presence of two different cell populations, one synthesizing embryonic chains and the other synthesizing adult hemoglobins. Only the embryonic cell population would then be sensitive to the hormonal and metabolic changes introduced under experimental diabetes. Figure 6 shows that experimental diabetes affects only the erythropoietin-dependent increase of embryonic globin chain synthesis at day 14 and has no effect on the adult-type hemoglobins. The mechanisms involved in this specific increase are not known. However, these observations are similar to the specific increase of fetal hemoglobins in man

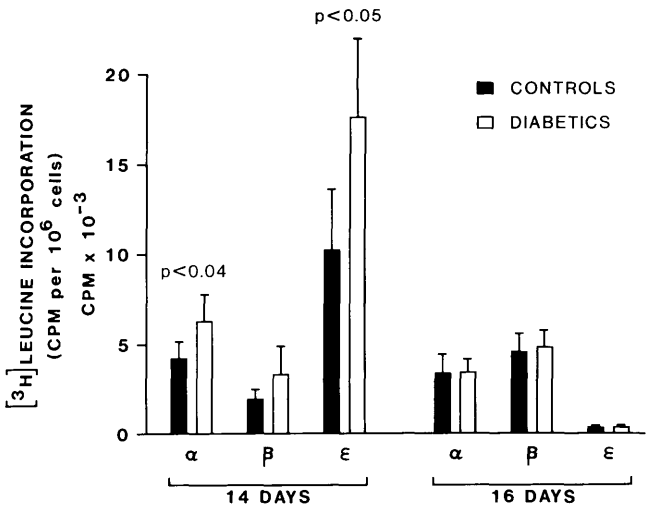

FIGURE 5. Comparison of the globin chain synthesis by liver cell cultures of 14- and 16-day-old fetuses of control and diabetic rats. The results are expressed as amount (cpm) of [^{3}H]-leucine incorporated per 10^6 cells after a 4-h incubation (see text). The fractions of alpha, adult beta, and embryonic chains correspond to HPLC peaks indicated in Figure 4. Alpha, fraction B; beta, fractions C–E; and embryonic, fractions F–I.

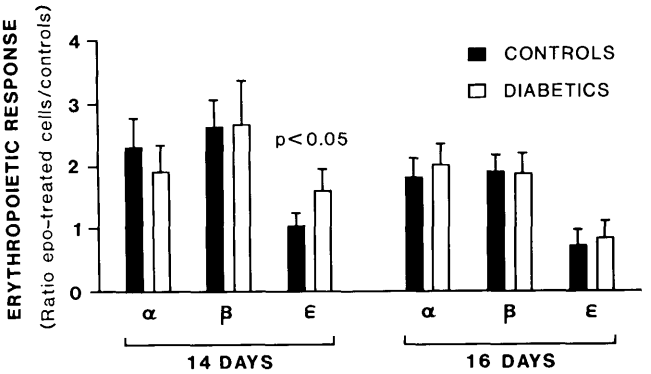

FIGURE 6. Effects of erythropoietin on globin chain synthesis. The cells were incubated for 20 h with 0.2 U/ml erythropoietin and the globin chain synthesis was measured as indicated in the text. The results have been expressed as ratios of erythropoietin-treated cells over control cell cultures. The erythropoietin-dependent increase in embryonic globin chain synthesis was statistically significant ($P < 0.05$).

under different conditions, including erythropoietic stress.[22] In these studies, we have examined the influence of experimental diabetes on erythropoietin-stimulated synthesis of embryonic- and adult-type globin chains rather than studying the role of erythropoietin itself, because in other studies we have shown that this hormone stimulates adult hemoglobin at both 14 and 16 days of gestation.[13] In fact all values >1.0 in Figure 6 indicate an erythropoietin-mediated increase in globin chain synthesis. Note that embryonic chain synthesis was stimulated by erythropoietin only in fetuses of diabetic mothers.

The hormonal or metabolic changes associated with the effect of experimental diabetes on erythroid cell functions described above remain to be elucidated. Although fetal hepatocytes are a major source of erythropoietin in mammals,[8] these cells play a very minor role, if any, in our culture system because they are virtually completely destroyed by the mechanical dispersion technique employed for cell preparation. Therefore, it is very unlikely that the differences observed between the control and diabetic animals is a consequence of differential survival of hepatocytes, which produce erythropoietin in larger amounts and act on the erythroid cells in culture. Hyperinsulinemia has been associated with high erythropoietin levels in hyperinsulinemic rhesus fetuses.[6] We have found high fetal insulin levels at days 19–22 of gestation,[16] but we were not able to measure this hormone at days 14 and 16 of gestation due to technical problems. However, circumstantial evidence[17,18] would suggest that experimental diabetes in rats could cause fetal hyperinsulinemia as early as 14–16 days of gestation. A direct role of insulin is suggested by the observation of significant uptake of labeled insulin by both the hematopoietic and parenchymal cells at day 17 of gestation[23] and by the stimulation of erythroid colony formation in fetal mouse liver by supraphysiologic concentrations (>10 nM) of insulin.[24] Moreover, we have found that insulin receptors are significantly decreased in both the fetal liver and lung at days 20–21 of gestation in diabetic animals.[25] Others have found that glucocorticoid receptors are present in erythropoietic fetal liver cells but not in hepatocytes, and there is a significant decrease in the number of receptor sites per cell.[26,27] We have found marginal differences in the binding capacity of glucocorticoids between days 19 and 21 of gestation in fetuses of control and diabetic rats,[16] but the fetal liver at this stage is transformed from a predominantly erythropoietic tissue to one primarily composed of hepatocytes. Therefore, it is still possible that, at an earlier gestational period (days 12–17), the binding to glucocorticoids in fetal liver cell cultures of control and diabetic rats is different. Both insulin and glucocorticoids may modulate the changes in erythropoietic response, and the role of these hormones is under further investigation.

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