Lack of Teratogenic Effect of Brief Maternal Insulin-Induced Hypoglycemia in Rats During Late Neurulation

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We have previously shown that 1 h of maternal insulin-induced hypoglycemia is teratogenic to rat embryos during the initial stages of neurulation, when they are dependent on uninterrupted glycolysis (day 9.5–9.7 of development). To determine whether this vulnerability persists in later stages of neural tube and cardiac development, we infused insulin into 16 conscious pregnant rats for 1 h beginning after embryos had developed the capacity for aerobic glucose metabolism (day 10.6 of development). Half of the pregnant animals were allowed to become hypoglycemic (44 ± 2 mg/dl) during the insulin infusions. The other half received glucose infusions to maintain normoglycemia (130 ± 3 mg/dl). Normal plasma glucose levels were maintained in all animals after the insulin infusions. Embryos were examined on day 11.5 of development. At that time, 1 of 111 embryos from the normoglycemic group and 1 of 109 embryos from the hypoglycemic group were grossly malformed ($P > .5$). Means of embryo crown-rump length (4.15 ± 0.03 vs. 4.14 ± 0.03 mm), somite number (29.7 ± 0.1 vs. 29.8 ± 0.2), and total protein content (320 ± 5 vs. 326 ± 6 μg) were also similar in the two groups ($P > .5$). Thus, we could not detect an embryotoxic effect of 1 h of maternal insulin-induced hypoglycemia beginning at day 10.6 of development. This finding is in contrast to our prior demonstration that a similar period of hypoglycemia occurring earlier in neurulation (day 9.7) caused growth retardation and developmental anomalies in embryos. Our results indicate that the teratogenic effects of brief maternal insulin-induced hypoglycemia in vivo do not extend past early organogenesis in rats. Based on these findings and on clinical data indicating that maternal hypoglycemia is not teratogenic during later portions of the first trimester, we suggest that efforts to determine whether maternal insulin-induced hypoglycemia is teratogenic in humans should focus on the first 3 wk of intrauterine development. *Diabetes* **38:1063–66, 1989**

Glucose 1 mM = 18 mg/dl

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Poorly regulated maternal diabetes during the first 6–8 wk of pregnancy is associated with an increased risk of congenital anomalies in infants (1–4). Improved blood glucose regulation before and during early pregnancy appears to reduce this risk (5,6). However, the extent to which glycemic control must be improved to minimize the risk to the developing embryo is unknown. Unlike late pregnancy, when even a small elevation of maternal glucose (i.e., gestational diabetes) is associated with fetal and neonatal complications (7–11), it is not clear that mild hypoglycemia during early pregnancy is associated with an increased risk of anomalies (2,3). On the other hand, animal studies indicate that maternal insulin-induced hypoglycemia, which is a frequent consequence of attempts at very strict glycemic control (12,13), may be harmful to mammalian embryos. Sadler and Horton (14) initially reported that serum from hypoglycemic rats was teratogenic to mouse embryos grown in vitro for 24 h. We subsequently developed an in vivo system with which we could examine the effects of shorter periods of hypoglycemia in a more physiological setting. We found that 1 h of maternal hypoglycemia during the initial stages of neural tube development caused growth retardation and developmental anomalies in rat embryos (15). As initially proposed by Freinkel et al. (16,17), this effect may have been due to the dependence of embryos on glycolysis during early organogenesis. If this is true, the toxic effects of maternal hypoglycemia should be reduced or absent once embryos have developed the capacity for aerobic glucose metabolism (16,17). This study was carried out to test our prediction with the same in vivo model that we initially used to dem-

onstrate the toxic effects of brief maternal hypoglycemia during early neurulation.

RESEARCH DESIGN AND METHODS

Normal male and virgin female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). This is the same rat strain that we used in our previous studies of the effects of maternal hypoglycemia on embryogenesis (15). Mating was accomplished by overnight housing of females with males and confirmed by the presence of sperm in a vaginal smear the following morning. Midnight of the night of mating was designated day 0 of embryogenesis; the following 24-h period was called day 1 of gestation (18). Pregnant females were housed singly with free access to water and pelleted food (Purina Rat Chow, Ralston Purina, St. Louis, MO).

Infusions. In the afternoon of the 7th day of gestation, each pregnant female underwent placement of an indwelling jugular venous catheter while under anesthesia. The distal tip of the catheter was advanced to the level of the right atrium; the proximal end was tunneled subcutaneously, externalized over the occiput, and filled with a solution containing povidone (PVP-40, Sigma, St. Louis, MO) and heparin (0.5 g PVP and 100 U porcine heparin/ml of 0.9% saline) until the day of infusions.

In the morning of the 10th day of embryogenesis, at least 4 h before infusions, bilateral tail vein infusion catheters were inserted percutaneously while animals were briefly restrained. The catheters and the distal tail were drawn through a hole in the cage and secured there with adhesive tape. This arrangement provided separate insulin and glucose infusion sites while allowing animals freedom to move about during infusions.

Primed (45 mU/kg body wt) continuous (10 mU · kg^{-1} body wt · min^{-1}) infusions of crystalline human insulin (Humulin, Lilly, Indianapolis, IN) were administered to 12-h-fasted animals for 1 h beginning at day 10.6 of embryo development. Plasma samples were obtained before, at 10-min intervals during, and for 80 min after the insulin infusions. Samples were analyzed immediately for glucose, and plasma from the 0-, 20-, 40-, 60-, 80-, 100-, and 120-min samples was stored at −20°C for subsequent analysis. Based on the immediate glucose determinations, half of the animals received an infusion of D-glucose (10% wt/vol in water) at a rate sufficient to maintain plasma glucose at the preinfusion level (normoglycemic animals, $n = 8$). The remaining eight animals were allowed to become hypoglycemic during the insulin infusions but were returned to normoglycemia with a glucose infusion immediately thereafter. Glucose infusions were maintained in all animals until the glucose-lowering effects of insulin had dissipated. Animals remained in tail restraints with free access to food and water after the infusions.

Embryo analysis. At day 11.5 of embryogenesis, pregnant animals were anesthetized with ether, and uteri were excised and placed in normal saline in a petri dish. During direct visualization through a dissecting microscope, fetuses were excised, and embryos were freed of surrounding decidual tissue and investing membranes. Each embryo was examined to determine whether the morphology of the brain spheres, neural tube, heart, optic and otic vesicles, limb buds, branchial arches, and axial curvature conformed to that expected at day 11.5 of development (19,20). Individual crown-rump length and somite number were also determined. These examinations were performed by a person who was unaware of the experimental group from which the embryos came. After examination, each embryo was placed in 0.5 N NaOH for subsequent measurement of total protein content.

Materials and analytical methods. Insulin for infusions was diluted in a sterile solution containing 0.01 ml autologous plasma/ml of 0.9% saline. Plasma glucose was measured by a glucose oxidase method (Beckman Glucose Analyzer II, Fullerton, CA). Plasma immunoreactive insulin was measured by a double-antibody radioimmunoassay with human insulin standard and guinea pig anti-porcine insulin antibody (both purchased from Novo, Copenhagen). The protein content of embryo digests was determined by the method of Lowry et al. (21).

Statistical analysis. Data are presented as means ± SE. Intergroup differences in the prevalence of morphologically abnormal embryos and resorbed conceptions were assessed by Fisher's exact test. Other comparisons were performed with unpaired t tests.

RESULTS

Insulin infusions produced prompt and sustained hypoglycemia in the absence of a concomitant glucose infusion (Fig. 1). The mean of plasma glucose in the hypoglycemic group fell to 42 ± 4 mg/dl by 20 min after the start of the insulin infusion and remained at 43 ± 5 mg/dl by 60 min. Animals in the normoglycemic group were maintained at approximately preinfusion glycemia during the same period. Glucose infusions restored normal plasma glucose levels in the hypoglycemic group within 20 min after the insulin was stopped. Glucose was kept normal in both groups until the glucose-lowering effects of insulin wore off (range 30–50 min after the insulin infusions).

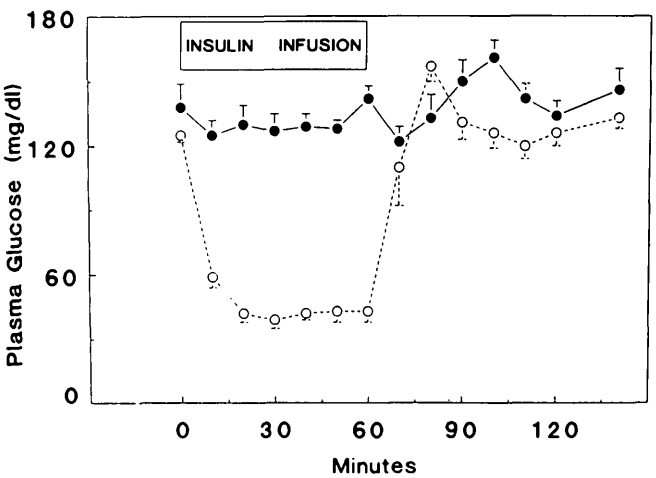

FIG. 1. Plasma glucose levels during and after 1-h insulin infusions into pregnant rats beginning on day 10.6 of embryogenesis (0 min). Eight animals were allowed to become hypoglycemic during insulin infusions but were returned to normal glucose levels immediately thereafter (○). Remaining 8 animals received glucose infusions to maintain euglycemia during and after insulin infusions (normoglycemic, ●).

Plasma insulin patterns were similar in the two experimental groups during and after the insulin infusions. The mean of 20-, 40-, and 60-min insulin values was 294 ± 36 μU/ml in the hypoglycemic group, similar to the mean of 304 ± 39 μU/ml in the normoglycemic group ($P > .5$). Insulin returned to a value not different from basal within 40 min after the insulin infusion in both groups.

The eight gravida from the normoglycemic group yielded 111 embryos for analysis on day 11.5. This was similar to the 109 embryos from the eight hypoglycemic animals. The mean number of resorbed conceptions did not differ significantly between the two groups (5 vs. 3; $P > .4$).

One embryo from each group was grossly malformed. The abnormal embryo from a normoglycemic mother had multiple deformities, including a malformed forebrain, slitlike optic vesicles, an inverted axial curvature, and small irregular somites. The abnormal embryo from the hypoglycemic group was also multiply malformed, with an open anterior neural tube, malformed brain spheres, absent optic vesicles, and irregular somites. The frequency of malformed embryos in each group was similar to the 1–2% rate of spontaneous malformations that we routinely observe in embryos from normal animals of this strain. Therefore, exposure to 1 h of maternal hypoglycemia beginning at day 10.6 of embryogenesis did not appear to cause gross malformations in embryos.

More sensitive measures of embryo well-being were also unaffected by the period of maternal hypoglycemia used in this study (Table 1). Mean crown-rump length (4.15 ± 0.03 vs. 4.14 ± 0.03 mm; $P > .5$), somite number (29.7 ± 0.2 vs. 29.8 ± 0.1; $P > .5$), and total protein content (320 ± 6 vs. 326 ± 5 μg; $P > .5$) of embryos were virtually identical between the normoglycemic and hypoglycemic groups. Thus, we were unable to detect any adverse effects on embryos resultant from exposure to 1 h of maternal hypoglycemia beginning at day 10.6 of development.

DISCUSSION

The findings of this study are in distinct contrast to our previous demonstration that 1 h of maternal insulin-induced hypoglycemia is teratogenic to rat embryos during the initial stages of organogenesis (day 9.5–9.7 of development; 15). We cannot attribute the difference to the hypoglycemia that we achieved in this study because the duration, nadir, and mean of glucose levels during the insulin infusions were virtually identical to those of our prior study (15). Rather, we believe that the difference is the result of a change in the susceptibility of embryos to glucose deprivation between days 9.5 and 10.6 of development. When neurulation begins (day 9.3–9.5; 20), rat embryos metabolize a significant proportion of available glucose to lactate (22,23). This process, which is relatively energy inefficient, is supplanted by a greater proportion of aerobic glucose metabolism coincident with the development of the yolk sac and allantoic placentas and their functional circulations (day 10.3–10.5; 22,23). Freinkel et al. (16,17) have shown that relatively small reductions in the flux of glucose through glycolysis between days 9.5–10.5 of development disrupts organogenesis in rat embryos (the "honeybee syndrome"). This vulnerability, which may be even more pronounced before the start of neurulation (24), abates after day 10.5, presumably because the development of aerobic glucose metabolism frees embryos from their critical dependence on glycolysis (16,17). In light of these facts, our finding that embryos are no longer vulnerable to brief hypoglycemia after day 10.5 of development supports the proposition that glucose deprivation per se mediated the teratogenic effects of maternal hypoglycemia that we previously observed before day 10.5 (15).

Our findings are in agreement with the in vitro studies of Akazawa et al. (25), who showed that serum from hypoglycemic animals was teratogenic to rat embryos between days 9.5 and 10.5 of development but not between days 10.5 and 11.5. Sadler and Horton (14), Sadler and Hunter (26), and Ellington (27) have also shown that serum from hypoglycemic animals is teratogenic to rodent embryos during neurulation, although the former group reported effects during both the early and late stages of neurulation in the mouse. Our in vivo system allowed us to examine two aspects of maternal hypoglycemia that were not addressed by these in vitro studies. First, we were able to focus on relatively brief hypoglycemia. The short duration of hypoglycemia that we used in this study may explain why we did not find embryotoxic effects of maternal hypoglycemia during late neurulation, whereas Sadler and Hunter (26) did. Second, hypoglycemia in vivo occurs in association with maternal hormonal, thermoregulatory, and cardiovascular responses that are not mimicked in vitro. Because these maternal responses can be expected to occur during all stages of organogenesis, our findings suggest they are not an important component of the toxic effects of brief hypoglycemia during early neurulation in vivo. However, we cannot wholly exclude the possibility that embryos respond differently to some aspect of the maternal counterregulatory response at day 9.5 than at day 10.6 of development.

Despite differences in methodology, the in vitro studies discussed herein and our work in vivo clearly indicate the teratogenic potential of maternal hypoglycemia in rodents. Although these findings clearly cannot be extrapolated to human pregnancy, they do suggest that pregnancies complicated by maternal diabetes be carefully examined for a relationship between maternal insulin-induced hypoglycemia and birth defects. To date, such a relationship has not

TABLE 1
Characteristics of rat embryos

Maternal group	*n*	Malformed embryos (*n*)	Crown-rump length (mm)	Somites (*n*)	Protein content (μg)
Normoglycemic	111	1	4.15 ± 0.03	29.7 ± 0.2	320 ± 6
Hypoglycemic	109	1	4.14 ± 0.03	29.8 ± 0.1	326 ± 5

Normoglycemic and hypoglycemic refer to maternal plasma glucose levels maintained during 1-h insulin infusions beginning at day 10.6 of embryogenesis. Embryos were examined at day 11.5 of development as described in RESEARCH DESIGN AND METHODS. No differences between group mean values reached statistical significance at $P = .05$.

been demonstrated. However, the results presented here suggest that clinical studies that have examined the relationship between maternal diabetes and birth defects have not been optimally designed to detect an effect of maternal hypoglycemia on development. Neurulation in humans begins at approximately day 18 of embryogenesis; a functional circulation is present by day 21–22 (28–30). If human embryos exhibit a vulnerability to brief maternal hypoglycemia that is similar to that of rodent embryos, then our findings indicate the effect should not be operative after the first 3 wk of development (31). None of the existing reports that have failed to detect an association between maternal hypoglycemia and birth defects in human diabetic pregnancies has focused on this early period (6,32,33). Thus, whether maternal insulin-induced hypoglycemia holds a teratogenic potential for human embryos remains a question open to careful clinical investigations that should focus on the first 3 wk of pregnancy.

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