Induction of Cytosolic Phosphoenolpyruvate Carboxykinase in the Ovine Fetal Liver by Chronic Fetal Hypoglycemia and Hypoinsulinemia¹

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ABSTRACT. Fetal glucose production has been observed in the chronically hypoglycemic, hypoinsulinemic (HH) fetal lamb. The purpose of this study was to test the hypothesis that induction of hepatic gluconeogenic enzymes occurs in this condition. The activities of both mitochondrial and cytosolic phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-diphosphatase, and glucose-6-phosphatase, three key enzymes of gluconeogenesis, were determined in fetal sheep liver from HH lambs and controls (CONT). Pregnant ewes were maintained chronically hypoglycemic by continuous hyperinsulinemic clamps from approximately 80 d of gestational age (53% of gestation) for 6 wk. Fetuses (gestational age: HH = 136 ± 2.6 , CONT = 133 ± 3.7 d) were maintained chronically hypoglycemic [HH = 0.51 ± 0.05 versus CONT = 1.22 ± 0.11 mmol/L (9.2 ± 1.0 versus 21.9 ± 11.9 mg/dL)] and hypoinsulinemic (HH = 3.3 ± 0.6 versus CONT = 12.0 ± 2.2 μ U/mL) and delivered by cesarean section after measurement of fetal glucose production rate. Hepatic cytosolic PEPCK was 6.0 ± 1.4 nmol/min/mg protein in CONT and 19.7 ± 2.5 in HH lamb ($p < 0.05$), whereas mitochondrial PEPCK was not different between the two groups. Neither fructose-1,6-diphosphatase or glucose-6-phosphatase activities nor plasma glucagon levels were different between groups. These results suggest that chronic fetal hypoglycemia and hypoinsulinemia prematurely induce hepatic cytosolic PEPCK in the fetal lamb. The observed fetal glucose production in the HH fetal lamb may be due to gluconeogenesis. (*Pediatr Res* 33: 493–496, 1993)

Abbreviations

PEPCK, phosphoenolpyruvate carboxykinase
G-6-Pase, glucose-6-phosphatase
HH, hypoglycemic, hypoinsulinemic
FDPase, fructose-1,6-diphosphatase

eventually develop gluconeogenic potential to maintain glucose homeostasis. Several studies have demonstrated that the development of hepatic gluconeogenesis is correlated with the appearance of cytosolic PEPCK (EC 4.1.1.32), the rate-limiting enzyme in gluconeogenesis (2). Several investigators (2–4) have attempted to prematurely induce hepatic PEPCK specific activity and gluconeogenesis *in vivo* in the fetus. However, despite induction of hepatic cytosolic PEPCK specific activity and mRNA accumulation, significant fetal glucose production has rarely been demonstrated until very close to term, coincident with large increases in cortisol production by the fetal adrenal gland (2, 5).

The chronically HH fetal lamb is a growth-retarded fetus that has been shown to have significant fetal glucose production (6). Because the HH fetal lamb is growth-retarded and has little hepatic glycogen, this observed fetal glucose production may be due to gluconeogenesis (6). Study of the gluconeogenic potential in such a model is important, as human infants with intrauterine growth retardation are frequently hypoglycemic, and limited gluconeogenesis has been suggested as one possible cause. We designed this study to determine the effect of chronic fetal hypoglycemia and hypoinsulinemia on the specific activities of PEPCK isoenzymes in the liver and kidney and the enzymes FDPase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) and G-6-Pase (EC 3.1.3.9) in the liver. We hypothesized that hepatic cytosolic PEPCK specific activity (the rate-limiting enzyme of gluconeogenesis) would be prematurely increased in the HH fetal lamb.

MATERIALS AND METHODS

Materials. Chemicals were of highest grade available and were purchased from Sigma Chemical Co. (St. Louis, MO). ¹⁴C-NaHCO₃ (3.4 mCi/mmol) was from New England Nuclear (Boston, MA).

Animal preparation. Experiments were conducted in pregnant Columbia-Rambouillet ewes. The ewes were fasted for 2 d before surgery. An initial limited surgery was performed at approximately 80 d of gestation (53% of gestation) under i.v. pentobarbital (5.0 mg/kg) or ketamine (12 to 15 mg/kg) sedation. Two maternal catheters for infusion were placed into a femoral vein through a left groin incision, and a single catheter for sampling of arterial blood was placed into the femoral artery. At approximately 115 d of gestational age, a second surgery was performed under i.v. pentobarbital sedation (5 to 15 mg/kg) and spinal anesthesia (tetracaine HCl 6.0 mg in hypertonic glucose) or with i.v. ketamine (12 to 15 mg/kg) and intramuscular acetylpromethazine (1 mg/kg) anesthesia. After a midline laparotomy, a catheter for sampling uterine venous blood was placed into the main uterine vein draining the pregnant uterine horn. Then, a

Hepatic gluconeogenesis does not occur at significant rates in the unstressed fetal sheep (1). After birth, the newborn must

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standard hysterotomy was performed, and catheters for fetal infusions were placed into fetal femoral veins via hindlimb pedal veins. Additional catheters for fetal blood sampling were placed into the fetal abdominal aorta via a hindlimb pedal artery and, by a direct method, into the common umbilical vein via one of the two umbilical veins at the base of the umbilical cord. All catheters were tunneled s.c. through a flank incision on the ewe and kept within a plastic pouch attached to the ewe's skin. The catheters were flushed every day with heparinized 0.9% (wt/vol) sodium chloride (150 U heparin). Each ewe was allowed to recover postoperatively for 2 to 3 d until behavior, food intake, and water intake were normal. The ewes were kept in carts and given an *ad libitum* diet of alfalfa pellets, water, and mineral supplements. Intramuscular B-complex vitamins (Vedco, St. Joseph, MO) were given every week to the ewe. The ewes were housed in a room with the temperature controlled between 10 and 20°C. At least two sheep were always housed together for company (6, 7).

Study design. One group of eight ewes was used for control studies (mean gestational age, 133 ± 3.7 d). This group did not receive any i.v. infusions except at the time of study. A second group of nine ewes (HH: gestational age 136 ± 2.6 d) received a continuous infusion of insulin i.v. for approximately 8 wk (80 to 135 d gestational age). Maternal arterial blood was sampled daily or twice daily for measurement of glucose and insulin concentration. The maternal arterial whole-blood glucose concentrations were used to adjust the insulin infusion rate to achieve a desired arterial whole-blood glucose concentration of approximately 1.4 mmol/L (25 mg/dL), representing an estimated reduction of 50% from normal. After fetal catheterization at 115 d gestation, it was observed that the maternal hypoglycemia also produced an approximately proportional decrease in fetal glucose and insulin concentrations (6).

Before autopsy, a metabolic study was performed. The maternal insulin infusion to maintain maternal and fetal hypoglycemia at the chronically produced level was continued during the study. Antipyrine was infused by a primed, constant infusion method into the fetus to measure umbilical blood flow by the transplacental steady-state diffusion technique (8). [U - ^{14}C]-glucose (332 mCi/mmol, ICN Biomedicals Inc., Costa Mesa, CA) was mixed with the antipyrine to deliver a primed constant infusion of tracer glucose to measure fetal glucose utilization (9). After 120 min of infusion, blood was sampled simultaneously from the umbilical arterial and venous catheters at four times separated by 15- to 20-min intervals. This blood was used to measure blood concentrations of glucose, tracer glucose, and antipyrine, blood oxygen saturation and content, and plasma insulin and glucagon concentrations. After metabolic studies, the fetus was delivered by cesarean section with pentobarbital anesthesia (25 mg/kg). The ewe was then killed with a rapid i.v. infusion of CII euthanasia solution (Anthony Products, Acadia, CA). The fetal tissues were removed and the fetus was killed by exsanguination.

All aspects of the animal procurement (Nebekar Ranch, Santa Monica, CA), care, surgery, recovery, maintenance, study, and euthanasia were approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee.

Chemical and analytical methods. Whole-blood glucose concentration was measured by a glucose oxidase method (Sigma). Maternal and fetal plasma insulin concentrations were measured by a double antibody method using a kit from Cambridge Medical Diagnostics, Inc. (Billerica, MA) and ovine insulin standards from Eli Lilly (Indianapolis, IN). Interassay and intraassay variability was less than 8%. Maternal and fetal plasma glucagon were determined by RIA, specific for pancreatic glucagon, using a kit from ICN Biomedicals Inc. The intraassay variability was $\pm 8\%$, and the interassay variability was $\pm 12.5\%$.

Enzymatic analyses. Cytosolic and mitochondrial fractions were prepared from fresh liver and kidney tissue according to the method of Chang and Lane (10). Mitochondrial contamination of the cytosolic extract was routinely assayed by citrate

synthase (11). PEPCK was assayed by the ^{14}C -bicarbonate fixation assay of Chang and Lane (10). Results are expressed as nmol of product fixed per min per mg of protein. Maximal FDPase activity was assayed by the method of Mazzotta and Veneziale (12) with results expressed as nmole of product formed per min per mg of protein. Protein was determined according to the method of Lowry (13) with BSA as the standard. G-6-Pase was assayed on glucose-free crude liver extracts by the spectrophotometric assay of Gierow and Jergil (14). Briefly, after homogenization in 0.35 M sucrose, 10 mM Tris, pH 7.4, the extract was centrifuged at $4000 \times g$ at 4°C for 15 min. The endogenous glucose was removed from the supernatant by passage on a Sephadex G25 column (Pharmacia, Piscataway, NJ). Parallel spectrophotometric determinations were performed without the addition of glucose-6-phosphate to confirm the absence of glucose (14). Results are expressed as nmol of product formed per min per mg of protein, assuming 2 nmole of NADH per nmol of product formed.

Statistics. Values are reported as mean $\pm$ SEM. Differences among groups were analyzed by *t* test with significance at $p < 0.05$.

RESULTS

Chronic maternal hyperinsulinemic, hypoglycemic clamps resulted in a reduction of mean fetal glucose and insulin concentration compared with control animals (Table 1). As shown in Table 1, the HH fetal lambs were smaller when compared with control and had reduced liver weight reflecting the intrauterine growth retardation of the HH fetal lamb. There was a significant reduction in net umbilical glucose uptake in the HH fetal lambs and a trend toward a lower rate of glucose utilization (Table 2).

Table 1. Fetal growth parameters, blood glucose, and plasma insulin and glucagon levels in control and HH fetal sheep*

	Control (n = 8)	HH (n = 9)
Fetal wt (g)	3190 ± 190	$2640 \pm 200^\dagger$
Fetal liver wt (g)	102 ± 7	$76 \pm 7^\dagger$
Mean fetal glucose [mmol/L (mg/dL)]	1.19 ± 0.11 (21.9 ± 1.9)	0.51 ± 0.06 (9.2 ± 1.0) ‡
Mean fetal insulin [pmol/L (μ U/mL)]	86 ± 16 (12.0 ± 2.2)	24 ± 4 (3.3 ± 0.6) †
Fetal plasma glucagon (ng/L)	208 ± 62	263 ± 110

* Insulin and glucagon values are from the day before lambs were killed. All values are mean $\pm$ SEM.

$^\dagger p < 0.01$ vs control.

$^\ddagger p < 0.001$ vs control.

Table 2. Metabolic parameters in control and HH fetal lambs*

Fetal metabolic parameters	Control (n)	HH (n)
Glucose uptake (mmol/kg/min)	0.25 ± 0.03 (6)	0.13 ± 0.02 (9) †
Glucose utilization (mmol/kg/min)	0.32 ± 0.04 (6)	0.23 ± 0.04 (6)
Glucose production rate (mmol/kg/min)	0.069 ± 0.030 (6)	0.116 ± 0.023 (6)
Arterial O ₂ saturation (%)	53.5 ± 2.8 (4)	54.4 ± 3.5 (9)
Arterial O ₂ content (mmol/L)	3.50 ± 0.21 (4)	3.06 ± 0.28 (9)
Umbilical O ₂ uptake (μ mol/kg/min)	329 ± 17 (4)	361 ± 17 (9)

* All values are mean $\pm$ SEM. Data available for only six of nine HH animals for glucose utilization and glucose production rate and four of six controls for O₂ saturation, content, and uptake.

$^\dagger p < 0.05$ vs control.

Glucose production rate was significantly greater than zero in the HH fetuses at a rate similar to that reported by us previously using a 2- to 3-wk period of insulin-induced hypoglycemia (6). Glucose production was also greater than zero in the control sheep due to data from two sheep in which glucose utilization rate was unexpectedly high. We do not have an explanation for the results in these two fetuses. Without their data, glucose utilization rate was 0.25 ± 0.03 mmol/kg/min (4.58 ± 0.52 mg/kg/min), not different from umbilical glucose uptake, representing a glucose production rate of 0.04 ± 0.03 mmol/kg/min (0.64 ± 0.49 mg/kg/min), not different from zero ($p > 0.05$).

Cytosolic PEPCK specific activity was significantly higher in the HH fetal liver as compared with controls. In contrast, there was no difference in the specific activity of mitochondrial PEPCK between the groups (Fig. 1). The hepatic cytosolic PEPCK specific activity in the HH fetal lambs was similar to that seen in 1-wk-old newborn lambs (mean of two values: 19 nmol/min/mg protein). Because the liver weight was reduced in the HH fetal lamb compared with control (Table 1), we compared total hepatic cytosolic PEPCK specific activity (specific activity in $\mu\text{mol}/\text{min}/\text{g}$ liver $\times$ g total liver wt), and there was still a significant increase in the HH fetuses (93 ± 11 $\mu\text{mol}/\text{min}$) compared with controls (26 ± 12 $p < 0.01$).

Cytosolic PEPCK is found in the liver, kidney, and, to lesser degree, in the intestine. Kidney cytosolic PEPCK may contribute to gluconeogenesis, although the hormonal and nutritional regulation of PEPCK in the kidney is different from that of the hepatic isoenzyme (15, 16). There was no significant difference in kidney cytosolic PEPCK between HH (13.4 ± 3.1 nmol/min/mg protein, $n = 7$) and controls (12.8 ± 2.1 , $n = 4$).

Mitochondrial PEPCK is an inner mitochondrial matrix enzyme (17). To confirm that the increase in hepatic cytosolic PEPCK in the HH group was not due to greater mitochondrial contamination in the HH group compared with control, citrate synthase, an inner mitochondrial matrix enzyme like mitochondrial PEPCK, was used. There was no difference in the percentage of citrate synthase contamination of the cytosolic hepatic fractions in the controls or HH fetuses ($4 \pm 4\%$ versus $9 \pm 5\%$). Thus, the increase in cytosolic PEPCK specific activity in the HH fetal lambs cannot be accounted for by an increase in contamination of the cytosolic fraction by the mitochondrial isoenzyme.

G-6-Pase is the last step in hepatic release of glucose. Hepatic G-6-Pase specific activity was not significantly different in the HH animals compared with controls (Table 3).

FDPase has been reported to be increased in fasting and is necessary for gluconeogenesis (18). FDPase specific activity was not significantly different between the HH and control fetal lambs (Table 3).

Cytosolic PEPCK specific activity and RNA levels are in-

Table 3. Effect of chronic fetal hypoglycemia and hypoinsulinemia on fetal hepatic FDPase and G-6-Pase*

	Control (n)	HH (n)
Maximal FDPase activity (nmol/min/mg protein)	19.2 ± 10.2 (5)	39.3 ± 8.3 (9)
G-6-Pase (nmol/min/mg protein)	6.2 ± 1.4 (8)	8.3 ± 2.1 (9)

* All values are mean $\pm$ SEM.

creased by glucagon and cAMP in the rat and in hepatoma cells (19, 20). Thus, plasma glucagon was analyzed to determine whether it correlated with the increase in cytosolic PEPCK specific activity. Plasma glucagon was not significantly different between the two groups (Table 1). The data in Table 1 for plasma glucagon are for the day before killing. If serial plasma glucagon measurements are included from the final 4 wk of the study period, there is still no difference between control (298 ± 38 ng/L, $n = 27$) and HH lambs (289 ± 28 ng/L, $n = 35$).

DISCUSSION

The production of chronic hypoglycemia and hypoinsulinemia in the fetal lamb with a chronic maternal hyperinsulinemic, hypoglycemic clamp results in an increase in hepatic cytosolic PEPCK specific activity to levels found in the 1-wk-old lamb but no changes in G-6-Pase or FDPase activity. The results of this study are in contrast to the results of Gruppiso *et al.* (21, 22) in fetal rats. In their studies, chronic HH fetal rats did not have an increase in hepatic PEPCK activity. There was an increase in G-6-Pase activity in one study (22) but not in the other (21). Girard *et al.* (4, 23) have demonstrated an increase in PEPCK in the rat fetuses of fasted, hypoglycemic mothers or phloridzin-treated, hypoglycemic mothers. The specific activity of PEPCK in these studies was still low (1.7 nmol/min/mg protein and 5.4 nmol/min/mg protein, respectively) compared with the levels in this study. Because the specific activity of hepatic cytosolic PEPCK is similar to that found in the 1-wk-old lamb and the 1-wk-old lamb has significant gluconeogenic potential, the enzymatic potential for gluconeogenesis exists in the HH fetal lamb, and the fetal glucose production observed in late gestation in the HH fetal lamb may be due to gluconeogenesis (5).

The mechanism of the up-regulation of hepatic cytosolic PEPCK in the HH fetal lamb is less clear. Glucagon, via its second messenger, cAMP, has been shown to increase hepatic cytosolic PEPCK specific activity, mRNA, and transcription rate in adult, late gestation fetal, and cultured adult rat hepatocytes (19). However, the effect of prenatal administration of glucagon to neonatal rats is dependent upon the gestational age. Before 18 d of gestation, there is no effect of glucagon on hepatic cytosolic PEPCK specific activity or mRNA steady state levels (24). After 18 d, PEPCK specific activity and mRNA are induced; however, fetal gluconeogenesis has not been demonstrated (24). In cultured fetal rat hepatocytes, glucagon or cAMP can increase PEPCK activity and mRNA as early as 14 d of gestation (25). However, because the fetal hepatocyte undergoes rapid maturation in culture, this may not reflect the situation *in vivo*. The fetal lamb has been reported to be insensitive to glucagon (26). However, studies supporting this observation were performed in the normal fetus, and not under conditions of chronic hypoglycemia and/or hypoinsulinemia, as observed in the HH fetal lamb. The lack of a difference in plasma glucagon concentrations between control and HH lambs would support the hypothesis that there is no direct relationship between plasma glucagon and cytosolic PEPCK induction in the fetal lamb liver in this model. Girard *et al.* (27) have suggested that the regulation of the induction of gluconeogenesis at birth is due to a decrease in the insulin to glucagon ratio. This would suggest that insulin and glucagon balance, not the absolute value of the individual hormones, might be important in the regulation of gluconeogenesis and cytosolic

Fig. 1. Comparison of hepatic cytosolic and mitochondrial PEPCK between HH and control fetal lambs.

PEPCK. There was a decrease in the insulin:glucagon molar ratio in the HH group. However, because this decrease was due solely to the decrease in plasma insulin in the HH animals, we were unable to separate the effect of hypoinsulinemia alone from that of the decrease in the insulin to glucagon ratio on cytosolic PEPCK. Evidence for the importance of the insulin:glucagon ratio and fetal glucose production also comes from other studies in fetal sheep in which insulin-regulatable glucose production was observed in fetal sheep made hypoinsulinemic but hyperglucagonemic by streptozotocin (28). In contrast, a much lower, almost negligible rate of glucose production was observed in fetal sheep made hypoinsulinemic but normoglucagonemic by pancreatectomy (29). Thus, in conditions with a low insulin to glucagon molar ratio, as in this model, fetal glucose production has been demonstrated (28). Future studies will need to address the mechanism of this increase in PEPCK in the chronically HH fetal lamb. One such mechanism might involve regulation by corticosteroids. Dexamethasone has been shown to increase cytosolic PEPCK activity and mRNA *in vivo* and *in vitro* (30–32), although this is controversial (25). Because the HH fetal lamb may be a stressed animal, it is possible that the induction of cytosolic PEPCK in these animals is in part due to an increase in corticosteroid secretion. Indeed, studies by Townsend *et al.* (5) and Fowden *et al.* (33) have demonstrated the importance of cortisol in promoting the capacity for glucose production in the late gestation fetal sheep.

Previous studies in the rat have suggested that fetal hypoinsulinemia and hypoglycemia can induce G-6-Pase specific activity (22); but this has not been a consistent finding (21). Our data for G-6-Pase are similar to the first study of Gruppuso *et al.* (21) in fetal rats. They found no effect of chronic maternal hyperinsulinemia/hypoglycemia on fetal hepatic G-6-Pase levels. Indeed, the level of hepatic G-6-Pase in the late-gestation fetal lamb is close to the level in the postnatal lamb and is most likely sufficient for glucose release from the liver.

In conclusion, we have demonstrated the premature induction of hepatic cytosolic PEPCK in the HH fetal lamb. We suggest that the enzymatic requirements for fetal gluconeogenesis are present in the HH fetal lamb, and that gluconeogenesis may account for the fetal glucose production of the HH fetal lamb. The chronically HH fetal lamb may provide a model to study the ontologic regulation of hepatic cytosolic PEPCK and factors regulating fetal gluconeogenesis.

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