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0031-3998/85/1901-0032$02.00/0
PEDIATRIC RESEARCH

Vol. 19, No. 1, 1985
Printed in U.S.A.

Altered Growth, Hypoglycemia, Hypoalaninemia, and Ketonemia in the Young Rat: Postnatal Consequences of Intrauterine Growth Retardation

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ABSTRACT. We characterized some of the consequences of intrauterine growth retardation in rat pups growth retarded [small for gestational age (SGA)] due to bilateral maternal uterine artery ligation. Pups of sham and nonoperated (normal) mothers served as controls. SGA pups had significantly reduced body and carcass mass throughout the study while body mass did not differ between sham and normal pups after 4 days. Brain mass was similar in the three groups at any age, while at 21 days and later, SGA liver weight as % body mass exceeded that of sham or normals. At 21 days, a 48-h fast reduced plasma glucose significantly in SGA compared to sham and normal pups; SGA plasma insulin was decreased and glucagon increased. Hepatic phosphoenolpyruvate carboxykinase activity and glycogen content were similar among groups. SGA pups did have significantly reduced plasma alanine and elevated betahydroxybutyrate levels. No differences in the responses to fasting occurred at 28 or 35 days. These data indicate that intrauterine growth retardation has profound effects on postnatal growth and metabolism. (*Pediatr Res* 19: 32–37, 1985).

Abbreviations

SGA, small for gestational age
RIA, radioimmunoassay
BOHB, β-hydroxybutyrate
PEPCK, phosphoenolpyruvate carboxykinase

Received February 1, 1984; accepted July 19, 1984.
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Supported in part by grants from the American Diabetes Association, NIH MRP HD 11021, and RR 05370, and the Abra Anderson Fund.

The consequences of intrauterine growth retardation on postnatal growth and metabolic development are not well understood. Human SGA neonates born to women with placental insufficiency are likely to remain small during later life (28). The alterations in organ growth associated with this diminished postnatal growth pattern are not known. In addition, while it is well recognized that the SGA newborn is at risk for hypoglycemia (5), it is unclear whether they have later difficulty with glucoregulation.

To address these questions, we used the technique of maternal uterine artery ligation to induce intrauterine growth retardation in the rat (29). Limited data indicate that SGA rat pups remain smaller than controls during early life in a manner similar to human infants (23), but no information is available concerning their glucoregulatory capabilities during later life. For this reason, we characterized the growth of SGA pups raised under well-defined conditions during the first 35 days of postnatal life. Also, the pups were fasted to determine their glucoregulatory abilities. Our findings suggest that the postnatal consequences of intrauterine growth retardation include both altered growth and diminished glucoregulatory capacity.

MATERIALS AND METHODS

Animal techniques. Time-dated pregnant Sprague Dawley rats were purchased on day 10 of gestation from Harlan Laboratories, Madison, WI. The rats had been mated at 9–10 wk of age and weighed 160–190 g at time of surgery. We modified Wigglesworth's (29) unilateral uterine artery ligation technique to bilateral ligation. On day 18 of their 21-day gestation, we anesthetized the rats with an intraperitoneal injection of chloral hydrate (60 mg/100 g body weight). The abdomen was incised under sterile conditions, and the uterus was isolated. We ligated both the right and left uterine arteries with 3–0 silk suture, returned the uterus to the abdomen, and closed the incision with suture and wound

clips. The procedure takes approximately 30 min. For the sham group, we anesthetized rats at day 18 of gestation, incised the abdomen, isolated and removed the uterus in the same manner as the SGA group, but did not ligate the arteries. The second control group, referred to as normals, was comprised of pups whose mothers did not undergo anesthesia or surgery. Rats who received chloral hydrate remained anesthetized for 2 ± 0.5 h.

Postnatal growth. Since fetal growth is to a degree inversely related to litter size (27), we attempted to limit variability due to this factor by using only pups from litters of 8–12. In addition, for studies of growth and glucose homeostasis, we included and compared only pups from SGA, sham, and normal litters who were delivered within 5 h. For all studies, we reduced litter number to four by randomly picking pups from a litter. This was done to maximize the pups' access to nutrition until weaning.

For characterization of growth, corresponding groups of SGA, sham, and normal pups who were born at the same time were raised in litters of four until weaning at 21 days. In pups who were 20 days and older, we estimated solid food intake by determining the daily difference in rat food weight. Littermates were maintained in cages of four in an environmental temperature of 21° C. Animals were weighed daily and measured every 4 days.

Fasting studies. Corresponding groups of SGA, sham, and normal pups were fasted at 10, 21, 28, and 35 days. At 0800 on the day of the fast, mothers were removed from the cages of the 10- and 21-day-old pups, and food was removed from the cages of the older pups. All pups had access to water during fasting. The 10-day-old pups were fasted for 24 h because of their inability to withstand longer fasting. Only organs were obtained from these pups. In older pups, fasted for 48 h, we sampled blood from the warmed, cut tail tip preceding, and at 24 and 48 h after initiation of fasting. Blood was collected in heparinized tubes, diluted with aprotinin (Trasylol, 30:1 v/v), and the plasma was separated and frozen. The pups were killed by decapitation and dissected to obtain liver, kidneys, stomach and intestine, spleen, heart, carcass, (muscle, skeletal bones, skin, and fur), and brain (cerebral hemispheres and midbrain). Body and organ mass was determined in newborn pups who were removed from their mothers before they could nurse. Organs were blotted dry and drained of as much blood as possible before weighing. Organ weights were determined after 12 h drying at 40° C. The livers used for biochemical measurements were quickly removed, weighed, cut into pieces, frozen in liquid nitrogen, and stored at −70° C.

Metabolic measurements. Glucose was measured in all samples, and whenever possible, insulin and glucagon in the same samples. Plasma glucose concentrations were measured with a Beckman II Glucose Analyzer, plasma insulin with a double antibody RIA system employing rat insulin as a standard (Novo Laboratories, Copenhagen, Denmark) (2). Plasma glucagon concentrations were assessed using antibody 30K in a double antibody RIA system (26). The interassay coefficients of variation were 12% for insulin and 21% for glucagon. Plasma alanine (31) and BOHB concentrations were measured using the methods of Williamson *et al.* (32) and hepatic cytosolic PEPCK activity by the method of Pollack *et al.* (21). PEPCK measurements were performed within 1 wk of collection of liver. Hepatic glycogen was determined by an enzymatic technique (12) and protein (16) and DNA (2) by spectrophotometric methods.

Statistics. We used the Student's unpaired t test and analysis of variance for comparison of groups (24). All data are presented as the mean ± 1 SEM.

RESULTS

Growth. The weights, snout-rump, and tail lengths of SGA, sham, and normal pups differed significantly at birth (Fig. 1). SGA pups weighed 4.20 ± 0.72; sham, 4.96 ± 0.66; and normal

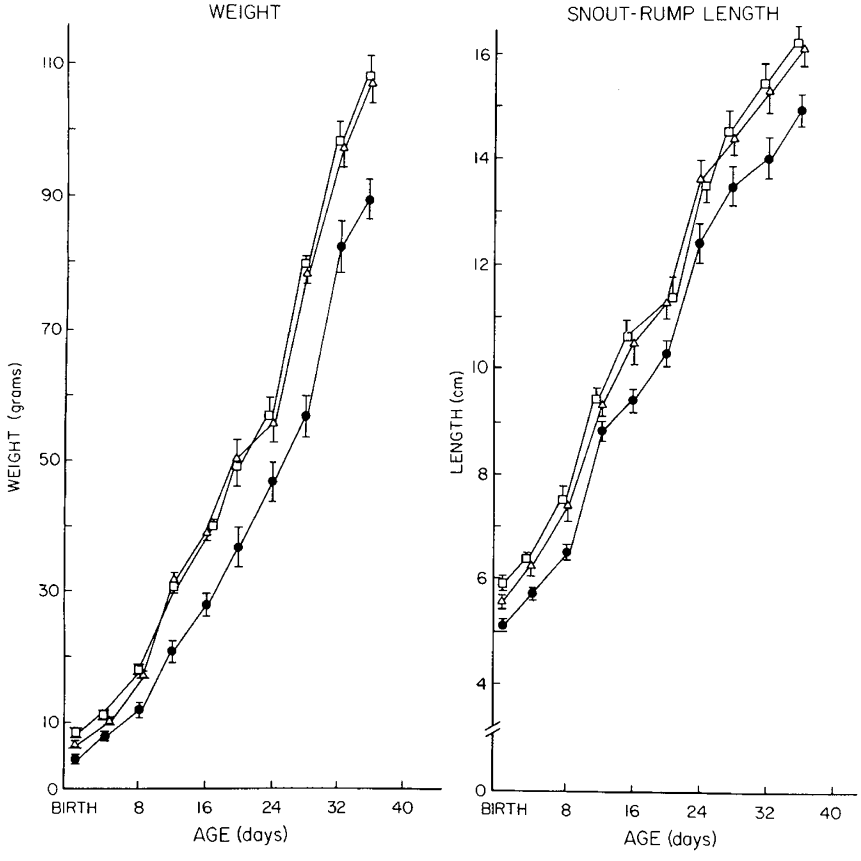

Fig. 1. Serial measurement of body weight and snout rump length of SGA (*circles*), sham (*triangles*), and normal (*squares*) rat pups. At birth, weight and length differed significantly between SGA, sham, and normal pups ($p < 0.01$). By 4 days, sham and normal pups no longer differed. SGA pups remained significantly lighter and shorter than sham and normal pups at all ages ($p < 0.01$ to <0.001).

5.82 ± 0.51 g, ($p < 0.001$, $n = 36$ in each group). Roughly 65% of pups had birth weights within 1 SD and 95% within 2 SD of the mean. Thus, birth weights were normally distributed when pups were considered in groups of four or as entire litters. The observation that the mean weight of the reduced litters was comparable to the mean weight of the total litter indicates that the selection of pups for growth studies was random and representative of the entire litter.

Throughout the first 35 days of life, SGA pups remained significantly lighter and shorter than sham and normal pups (Fig. 1). Sham pups attained the weight and length of normal pups by 4 days, and the subsequent growth of these groups did not differ. Snout-rump and tail length correlated significantly for each group at all ages. While males tended to be somewhat heavier and longer than females, these differences were not significant. Pups who were fasted and killed at different ages demonstrated the same growth patterns as those who were fed *ad libitum* the first 35 days. Although the rate of weight gain varied with age, weight gain did not differ between SGA, sham, and normal pups as suggested by the roughly parallel growth curves in Figure 1. Depending on age, pups of all groups demonstrated a fractional weight gain of 0.08–0.10 of the preceding day's weight. SGA pups ate significantly less solid food from 3–5 wk of age, but when food intake was expressed relative to body weight, no differences were apparent among the groups. Immediately following weaning, pups ate approximately 0.07–0.08 g food/g body weight. By 1 day after weaning, pups were eating 0.10–0.14 g food/g body weight.

Table 1 details the wet organ weight. The weight of brain, heart, kidneys, and spleen did not differ among groups at any age. At birth, carcass and liver weight differed between groups with values for SGA pups being significantly reduced, normals significantly increased, and shams, intermediate. Differences between the sham and normal pups were no longer significant by the 4th day of life. SGA pups had significantly reduced liver and carcass weight at all points. SGA liver weight was significantly reduced when considered as either overall wet (Table 1) or dry weight (Fig. 2). Liver, kidney, spleen, and heart were 75–80% water for pups of all ages. SGA pups tended to have slightly but not significantly increased body water at birth. In all groups, brain water content at birth, 4, and 10 days was significantly greater (85 ± 3%) than at later ages (79 ± 2%).

At birth, compared to normal pups, SGA and sham pups had slightly increased liver to body weight ratios. This difference between sham and normal pups disappeared by day 4 of life. By 10 days, the fraction of body weight attributable to liver was equivalent in all groups; however, by 21 days and at later points, SGA pups once again had significantly increased liver/body weight ratios related to sham or normal pups (Figure 2).

Hepatic DNA was significantly greater in SGA pups than sham or normals at 10 days and later ages (Table 2). Hepatic protein concentration was equivalent at 10 days in all groups but was significantly increased in SGA pups at 21, 28, and 35 days. Thus, the DNA/protein ratio was significantly increased at 10 days but not at later ages. Hepatic DNA content was greatest at birth (6.3–7.3 mg/g liver). This most likely represents hematopoietic function of the liver in the immediate postnatal period.

Fasting studies. With fasting, pups lost an average of 9% of

Table 1. *Organ mass of rat pups*

Age (days)	Brain (mg)			Liver (mg)			Carcass (g)		
	SGA	Sham	Normal	SGA	Sham	Normal	SGA	Sham	Normal
Birth	190 ± 50	191 ± 40	220 ± 40	219 ± 30*	251 ± 31*	280 ± 30	3.2 ± 0.1*	4.3 ± 0.1*	5.2 ± 0.1*
10	814 ± 58	856 ± 60	864 ± 70	606 ± 62*	766 ± 30	742 ± 31	160 ± 0.7*	21.0 ± 1.0	22.0 ± 1.5
21	1180 ± 88	1200 ± 70	1230 ± 96	1320 ± 90*	1540 ± 60	1540 ± 60	8.0 ± 3.0*	42.1 ± 2.0	41.7 ± 3.0
28	1300 ± 90	1320 ± 90	1360 ± 86	2060 ± 90*	2660 ± 90	2660 ± 90	40.7 ± 3.0*	61.2 ± 4.1	63.9 ± 4.0
35	1470 ± 100	1440 ± 88	1550 ± 92	3250 ± 130*	3720 ± 140	3750 ± 100	68.4 ± 4.0*	89.3 ± 5.0	92.0 ± 5.0

Each group was comprised of a minimum of 10 pups.
* Indicates difference ($p < 0.01$) from groups at the same age.

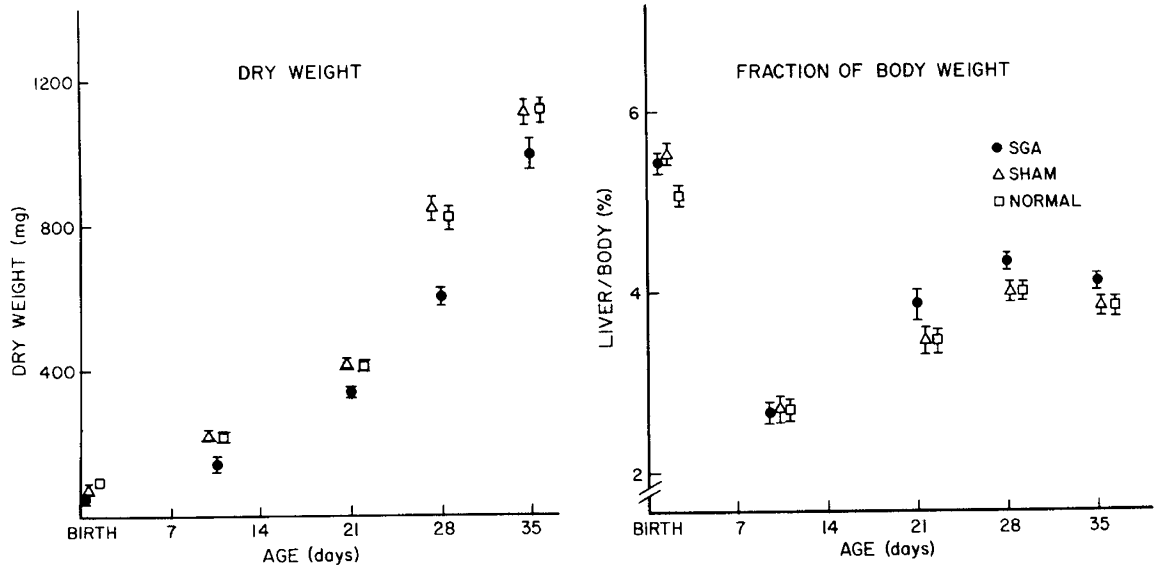

Fig. 2. Hepatic growth in SGA (*circles*), sham (*triangles*), and normal (*squares*) rat pups. At birth, liver weight differed significantly between all groups. SGA pups had significantly smaller livers throughout the study ($p < 0.01$ to <0.001). At birth, the fraction of body weight attributable to liver was greater in SGA and sham than normal pups. At 4 and 10 days, the fraction of body weight attributable to liver was equivalent; by 21 days and later, liver comprised a significantly greater fraction of body weight in SGA than in sham and normal pups ($p < 0.001$).

Table 2. *Hepatic growth*

Age (days)	DNA (mg/g liver)			Protein (mg/g liver)			DNA/protein		
	SGA	Sham	Normal	SGA	Sham	Normal	SGA	Sham	Normal
10	4.00 ± 0.50	2.76 ± 0.40	2.84 ± 0.38	19 ± 1.6	20 ± 1.4	20 ± 1.0	0.21 ± 0.07*	0.14 ± 0.07	0.14 ± 0.06
21	3.58 ± 0.39*	2.60 ± 0.30	2.84 ± 0.20	26.4 ± 1.0*	18.3 ± 1.5	21.8 ± 0.8	0.14 ± 0.06	0.14 ± 0.07	0.13 ± 0.05
28	3.60 ± 0.20*	2.60 ± 0.45	2.62 ± 0.23	26.0 ± 2.1*	19.6 ± 2.1	19.1 ± 1.4	0.14 ± 0.06	0.13 ± 0.06	0.13 ± 0.07
35	3.72 ± 0.24*	2.67 ± 0.50	2.68 ± 0.33	28.0 ± 2*	20.2 ± 2.4	20.6 ± 2.0	0.13 ± 0.07	0.13 ± 0.06	0.13 ± 0.05

Each group was comprised of a minimum 20 pups.
* Indicates difference ($p < 0.01$ or greater) from groups at the same age.

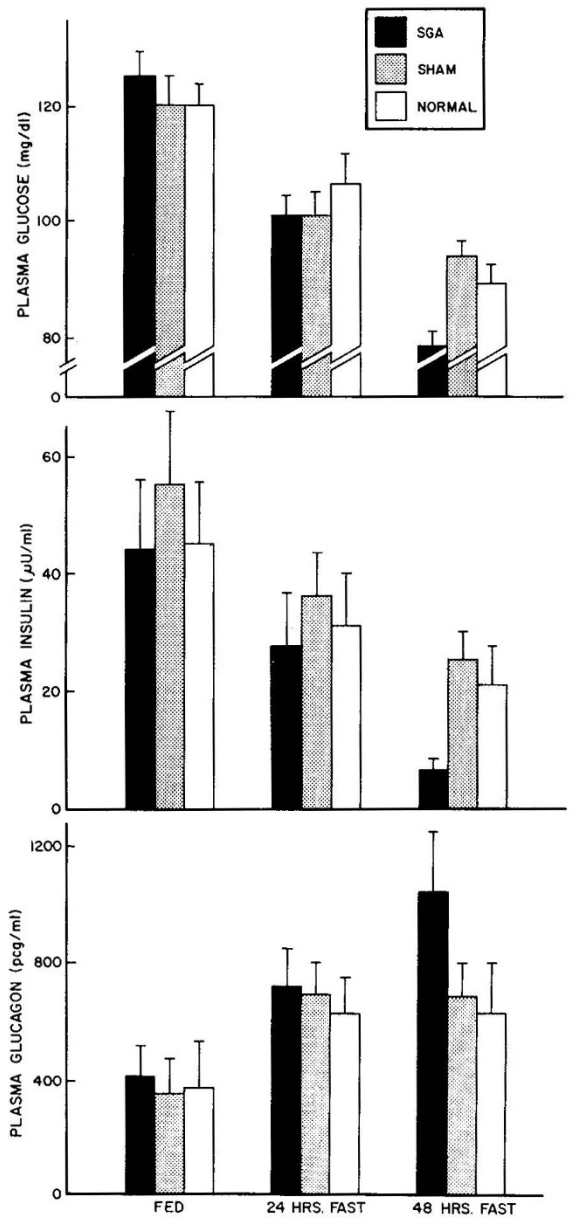

Fig. 3. Serial plasma glucose, insulin, and glucagon concentrations of GA, sham, and normal rat pups at 21 days of life. All pups had similar lasma concentrations of glucose, insulin, and glucagon prior to fasting. wenty-four and 48 h fasting were associated with significant reductions ı glucose and insulin and significant increases in glucagon for all pups. ollowing 48 h fasting, SGA pups had significantly diminished plasma ucose and insulin and greater glucagon concentrations than sham and ɔrmal pups ($p < 0.01$). A minimum of 20 pups comprised each group.

refasting weight after 24 h and 15% by 48 h. Plasma glucose ecreased with fasting for all pups. The most dramatic changes nd only differences between groups developed at 3 wk. SGA, ıam, and normal pups had similar plasma glucose concentra-

Table 3. *Hepatic PEPCK activity (μmol PEP/g liver/min)*

Age (days)	10	21	28	35
SGA	0.325 ± 0.020	0.531 ± 0.020	0.640 ± 0.103	0.670 ± 0.110
Sham	0.309 ± 0.060	0.540 ± 0.053	0.690 ± 0.095	0.680 ± 0.090
Normal	0.330 ± 0.041	0.580 ± 0.069	0.670 ± 0.100	0.630 ± 0.093

Each value represents measurements from a minimum of 15 pups. Values for pups of all groups at 10 days were significantly less ($p < 0.01$) than pups at later ages. Values for 21, 28, and 35 days do not differ.

tions before and 24 h after fasting; however, by 48 h, plasma glucose concentrations for SGA pups had decreased by 35% while values for shams and normals had decreased only by 20% (Fig. 3). Plasma insulin concentrations also decreased with fasting. After 24 h, no differences were observed among 3-wk-old SGA, sham, or normal pups; however, after 48 h fasting, plasma insulin concentrations were significantly lower in SGA than in sham or normal pups. The 48-h values for all three groups of pups were significantly less than prefasting values. Plasma glucagon levels increased with fasting. Three-week-old SGA pups had significantly greater plasma glucagon concentrations at 48 h than the sham or normal pups. Similar patterns of significant increases of plasma glucagon concentrations occurred at 4 and 5 wk with 24 and 48 h fasting; however, at these later ages, no differences between SGA, sham, and normals before or with fasting could be distinguished.

SGA, sham, and normal pups had equally low hepatic glycogen concentrations after 48 h fasting at 21 days (5.2 ± 0.3 *versus* 5.2 ± 0.4 *versus* 5.4 ± 0.2 mg/g liver, respectively). Similar values were noted after fasting at 28 and 35 days. In fed pups at these ages, hepatic glycogen concentrations did not differ between groups with values ranging from 9.9–19.7 mg/g liver.

Hepatic cytosolic PEPCK activity in SGA, sham, and normal pups did not differ at any age. Values for 21-, 28-, and 35-day-old pups were equivalent and significantly exceeded values for 10 day pups (Table 3).

Forty-eight hours fasting in 21- but not 28- or 35-day-old pups resulted in significantly decreased plasma alanine and elevated betahydroxybutyrate concentrations (Table 4). Plasma alanine and BOHB concentrations after 24 and 48 h fasting did not differ between SGA, sham, and normal pups at 28 or 35 days. Values for fed pups at these ages did not differ although the variability was greater for these measurements.

DISCUSSION

Our results indicate that SGA rat pups tend to remain significantly smaller than controls and also develop hypoglycemia with fasting during early postnatal life. SGA pups remained significantly smaller than sham and normal pups for the first 35 days. This difference is primarily attributable to diminished growth of carcass, *i.e.* muscle, adipose, and bone tissue, as indicated by its significantly decreased mass throughout the study. Of note, heart, kidneys, and spleen did not differ between groups. Similarly, gross brain growth did not differ between SGA, sham, and normal pups. Liver mass remained significantly reduced up to 35 days in agreement with earlier observations reporting limited hepatic growth to 20 days (23). In our study, however, the liver/

Table 4. *Plasma metabolic fuel concentrations (μmol/ml) in 21-day-old pups*

	Alanine			BOHB		
Group	SGA	Sham	Normal	SGA	Sham	Normal
Fed	0.240 ± 0.020	0.232 ± 0.053	0.212 ± 0.023	0.291 ± 0.023	0.266 ± 0.022	0.200 ± 0.020
24-H fast	0.190 ± 0.035	0.237 ± 0.062	0.229 ± 0.015	0.577 ± 0.092	0.488 ± 0.081	0.570 ± 0.047
48-H fast	0.140 ± 0.021*	0.220 ± 0.023	0.222 ± 0.028	1.186 ± 0.111*	0.800 ± 0.096	0.812 ± 0.094

* Significantly different from sham and normal values ($p < 0.001$).

body weight ratio in the SGA pups was increased at 10 days compared to sham and normal pups. This increase in relative liver growth rate resulted from both cellular hyperplasia and hypertrophy as suggested by the increase in both hepatic DNA and protein content. Others have not observed postnatal hepatic hypertrophy or hyperplasia (23, 25). This difference may represent our bilateral uterine artery ligation and our attempt at optimizing the pups access to nutrition by limiting litter number. In fact, we found that solid food intake with respect to body mass was equivalent for SGA, sham, and normal pups during the postweaning period.

We modified the method first described by Wigglesworth (29) by performing surgery at a later gestational age and ligating both uterine arteries. While pups from the nonligated uterine horn have been used for controls (11, 17, 19, 23, 29), we and others (13) have found considerable variation in the size of litter mates from the nonligated horn and, for this reason, chose to use pups of both sham and nonoperated mothers as controls. As observed by others (13), pups of sham-operated mothers are significantly smaller at birth than nonoperated mothers. This indicates that 2–2.5 h of maternal chloral hydrate anesthesia, abdominal incision, and manipulation of the uterus on day 18 of gestation are sufficient stresses to limit fetal growth during late gestation. This effect, unlike uterine artery ligation, is transient since the growth patterns of the sham-operated pups attained that of normals by the 4th postnatal day.

We designed our study to include groups of SGA, sham, and normal pups delivered within 4 h of each other so that comparisons could be made between corresponding groups. We also limited selection of pups by using only those from litters of 8–12 to diminish the effect of litter size on fetal growth. The observation that the mean weight of the reduced litters was comparable to the mean weight of the total litter indicates that this selection was random and representative of the litter. While the study design might have been improved by cross-fostering of SGA, sham, and normals, this could not be accomplished because of maternal rejection of pups from other litters.

While growth was altered throughout the 35 postnatal days, differences in glucose homeostasis were evident only at 21 days. At 3 wk, a 24-h fast resulted in similar changes in plasma concentrations of glucose, insulin, and glucagon in all groups and 48-h fasting resulted in hypoglycemia in the SGA pups. In addition, at 48 h, fasted SGA pups had significantly decreased plasma concentrations of insulin and increased concentrations of glucagon, *i.e.* appropriate glucoregulatory hormone responses to fasting.

Nonetheless, hypoglycemia occurred, indicating a defect in glucoregulation despite the appropriate hormone signals. To define the defect we measured hepatic PEPCK activity as representative of gluconeogenic capability because of its key role in gluconeogenesis in the newborn rat pup. During fetal life, PEPCK activity is virtually absent (1, 22); its activity increases approximately 25-fold during the first 24–48 h of life (1, 22, 33). Pyruvate carboxylase, fructose 1,6-diphosphatase and glucose-6-phosphatase (1, 22, 33, 34) are induced during later fetal life and their activities during neonatal life, although relatively limited compared to later life, are adequate to sustain gluconeogenesis. Thus, PEPCK is considered rate-limiting in the normal newborn rat. Our preliminary results suggest that the sequential increase in PEPCK activity characteristic of the normal newborn during the first 6 h of life is significantly delayed in SGA pups (3). These observations concur with studies demonstrating limited conversion of labeled substrate to glucose by the liver of SGA newborn rats (17). The demonstration of elevated plasma concentrations of gluconeogenic precursors and inability to convert exogenous alanine to glucose in SGA human newborns suggest that such a deficit exists in the human as well (30).

This developmental delay appears to be transient since we found similar values for hepatic PEPCK activity in SGA, sham, and normal pups at 10 days and at later ages. Our results and earlier reports that newborn SGA pups have appropriate activities of the other gluconeogenic enzymes at birth and normal activities of glucose-6-phosphatase and fructose1,6-diphosphatase activities up to 30 days of age (4, 14) suggest that gluconeogenic enzyme activity is most likely intact in the 3-wk-old SGA pup. The significant increase in hepatic PEPCK activity between 10–21 days in all three groups of pups represents the normal pattern of this enzyme during this period.

Inadequate glycogen stores and diminished glycogenolytic capability were not responsible for the development of hypoglycemia in the 3-wk-old SGA pup since in all groups fed pups had equivalent hepatic glycogen concentrations and fasted pups had equally low glycogen concentrations. This is consistent with the earlier observation of intact mechanisms for glycogen synthesis and mobilization in the SGA rat (19).

Limited mobilization of gluconeogenic substrate is most likely responsible for the inability of the SGA pup to maintain normoglycemia during prolonged fast at 3 wk. The significantly diminished plasma alanine concentrations indicate that limitation of this key gluconeogenic precursor was responsible for the development of hypoglycemia. Studies in laboratory animals and humans indicate that during fasting, skeletal muscle protein is preferentially converted to alanine which then becomes available for hepatic gluconeogenesis (7). Decreases in plasma alanine concentration with fasting have been reported in both laboratory animals and humans (6, 18). The diminished mean plasma alanine concentration in our study suggests that alanine release by skeletal muscle was limited in the 3-wk-old SGA pups although measurement of alanine flux would be necessary to confirm this impression.

Carcass mass in our SGA animals remained significantly diminished while gross brain growth was normal throughout the study. At 21 days, the disproportion between brain and muscle mass might have been responsible for the development of hypoglycemia. It may be that the glucose requirements of the brain far exceeded the ability of muscle mass to provide gluconeogenic substrate. The fact that 28- and 35-day-old SGA pups withstood fasting as well as controls suggests that with attainment of increased body and carcass mass, the disproportion in organ size in SGA pups was no longer an important factor causing hypoglycemia. However, the mechanism is not clear. The glucagon/insulin ratio was appropriately increased. It might be speculated that at 21 days, the SGA pup has mechanisms during fasting to brake the catabolism of already limited skeletal muscle protein for conservation of the limited muscle mass.

Ketosis was obvious with fasting in all three groups of pups, but was more pronounced in the hypoglycemic SGA animals. This accelerated ketosis indicates normal stimuli and mechanisms of free fatty acid mobilization. Our hypoglycemic, ketotic SGA rats at 35 days resemble infants with ketotic hypoglycemia.

This generally develops in children after the 1st yr of life (8). Many, although not all, children who develop ketotic hypoglycemia of infancy are SGA at birth suggesting a possible association between these conditions. While the mechanisms responsible for this disorder are far from understood, limited observations suggest that limitation of alanine for gluconeogenesis may be primarily responsible for the development of hypoglycemia (10, 20). This rat model may serve as a means for studying the pathogenesis of ketotic hypoglycemia of infancy.

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