Characterization of Carbohydrate Metabolism in the Isolated Fetal Rat Heart

Effects of Fasting and Alloxan Diabetes

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SUMMARY

Studies have been made on the effects of maternal fasting and alloxan-induced diabetes on fetal and maternal plasma glucose and insulin concentrations, as well as on fetal myocardial and hepatic glycogen concentrations. Maternal fasting for sixteen hours, ending seventy-two hours prior to sacrifice, had no effect on maternal glucose and insulin concentrations. Under these conditions fetal insulin and hepatic and myocardial glycogen concentrations were also unchanged; fetal plasma glucose increased [from 16±2 to 26±3 mg./100 ml. ($p<0.02$)]. A sixteen-hour maternal fast immediately preceding sacrifice significantly decreased maternal insulin and glucose concentrations. Fetal hepatic and myocardial glycogen levels and insulin concentrations were also decreased; fetal glucose again increased [from 35±1 to 43±1 mg./100 ml. ($p<0.001$)]. The regulation of glycogen synthesis and degradation in the fetal rat, and the importance of fetal insulin on fetal glycogen concentrations are discussed.

Maternal alloxan diabetes produced elevated glucose concentrations, unchanged insulin values, decreased hepatic glycogen and increased myocardial glycogen in the fetuses. Possible mechanisms for these findings are discussed. Fetal myocardial glucose uptake in vitro was not affected by either fasting or maternal diabetes.

The data suggest that, whereas near-term fetal rats do possess some ability to control their glucose and glycogen concentrations, manipulation of maternal nutrition by fasting or alloxan diabetes can significantly alter fetal carbohydrate metabolism. DIABETES *23*:662-68, August, 1974.

During the latter third of gestation, fetuses from many mammalian species store glycogen in a variety of organs.[34] The initiation and regulation of this fetal glycogen storage is poorly understood.

It has been previously demonstrated that fasting and diabetes significantly alter hepatic and myocardial glycogen storage[3,13,14,32] and myocardial glucose uptake[28,30] in the adult rat. The purpose of the studies herein reported was to characterize the effects of fasting and alloxan-induced diabetes on fetal myocardial glucose uptake and hepatic and myocardial glycogen storage. Fetal and maternal glucose and insulin concentrations were also measured. The results suggest that maternal nutrition and diabetes have significant effects on fetal glycogen storage.

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METHODS

Animals. Pregnant rats of known gestational age were obtained from the Charles River Breeding Laboratory and maintained on Purina Rat Chow. Gestational ages of the fetuses were confirmed by their weight.

Fasting. Animals always had free access to water. The fasts began at 4 p.m. and terminated at 8 a.m. the next day. Preliminary experiments on the effects of ether anesthesia on maternal and fetal glucose and insulin concentrations, and fetal hepatic and myocardial glycogen concentrations indicated that if this procedure was performed carefully, no anesthetic effect

was observed. In addition, each experiment was internally controlled. With the exception of the variable under investigation, experimental and control animals were thus treated identically as regards gestational age, surgical manipulation and anesthesia at sacrifice. Mothers bred on the same day were always used *within* each experiment. Subsequent differences in control values *between* experiments reflect different fetal ages at time of sacrifice.

Alloxan diabetes. Following a sixteen-hour fast, mothers were lightly anesthetized with ether and injected via the saphenous vein with a small volume of water containing either alloxan (60 mg./kg.) or 0.9 per cent NaCl. The alloxan was always made up immediately before use. The animals were then replaced in their cages with free access to food, 5 per cent dextrose, 0.9 per cent NaCl and water.

Induction of fetal diabetes was attempted by injection of alloxan (60 mg./kg. estimated fetal weight) intraperitoneally through the placental membranes; this procedure was performed under ether anesthesia following maternal laparotomy with sterile technic.[8] Controls were injected with 0.9 per cent NaCl. During some procedures alternate fetuses in the same mother were injected with alloxan or saline; in other experiments entire litters were injected with either substance. The results were identical and the data have been combined. After surgery the animals were allowed free access to food and water.

Both maternal and fetal diabetes were induced on day 18 of gestation, with sacrifice on day 21. Maternal survival was virtually 100 per cent; fetal reabsorption rates following intraperitoneal injections were 10 to 20 per cent and were equally distributed between alloxan- and saline-treated animals.

Analyses. The mothers were lightly anesthetized with ether, the fetuses were quickly removed by cesarean section, and then decapitated. Fetal blood was collected in pour plates containing a small amount of powdered heparin, or the hearts and livers were rapidly excised, frozen on dry ice, weighed and analyzed for glycogen as described below. Time of initiation of anesthesia to collection of appropriate specimens did not exceed five minutes; not more than thirty seconds elapsed between removal and decapitation of fetuses and freezing of organs. The heparinized blood from each litter of fetuses (eight to thirteen fetuses) was pooled and centrifuged; plasma was analyzed for glucose in duplicate by a glucose oxidase method (Glucostat, Worthington Biochemical, Freehold, New Jersey) and insulin in duplicate by radioimmunoassay.[44]

The frozen hearts and livers were placed in cold (4° C.) trichloroacetic acid and homogenized. Glycogen was then isolated by a modification of the method of Good, Kramer and Somogyi[19] previously described.[6] After hydrolysis of the glycogen the glucose was measured by glucose oxidase as above. The concentration of glycogen is expressed as μmoles of glucose equivalent per gram wet weight.

Glucose uptake. Fetal myocardial glucose uptake was performed as previously described.[7] Mothers were anesthetized with ether and the fetuses were removed by cesarean section. Fetal hearts were then removed and placed in 0.9 per cent NaCl at room temperature. After all hearts were collected they were blotted free of blood, weighed and placed in separate 12 x 75 mm. disposable glass tubes (Bellco Glass, Vineland, New Jersey) containing 0.5 ml. of Krebs-Ringer bicarbonate buffer to which had been added 0.0625 ml. of 16 per cent gelatin (Armour lot no. K188151) (final concentration of 2 mg./ml.), 0.2 μC of 1-14-C glucose (New England Nuclear, lot no. 522-29, 6.4 mc/mmole), and 3.5 μmoles of unlabeled glucose (final concentration 7 mM). "Glucagon-free" beef insulin was added in .01 ml. volumes in buffer, whereas 0.01 ml. buffer was added to the control tubes. The tubes were then gassed with 95 per cent oxygen to 5 per cent carbon dioxide and sealed with airtight caps (Capall, Scientific Products, Evanston, Illinois). Incubations were carried out at 37° in a Dubnoff metabolic incubator (100 cycles per minute).

Medium in the amount of 0.01 ml. was placed in 10 ml. of scintillation mixture made up as follows: 333 ml. Triton-X 100, 4 gm. 2, 5 Diphenyloxazole (PPO), 40 mg. 1, 4-bis-2-(4-methyl-5-Phenyl-oxazolyl)-Benzene (dimethyl POPOP) (Packard Instrument, Downers Grove, Illinois) and 667 ml. of toluene. The samples were counted in a Packard Model 4322 liquid scintillation counter at 56 per cent efficiency and with a counting error of 1 per cent or less. Glucose uptake was estimated by the following formula:

$$\text{Glucose uptake } (\mu\text{mole/gm.}) = \frac{(\text{Initial cpm/ml. media - Final cpm/ml. media}) \times (\text{volume of incubation media})}{(\text{Tissue wet weight in gm.}) \times (\text{cpm}/\mu\text{mole in the initial media})}$$

The largest absolute amount of glucose taken up was less than 8 per cent of the total glucose available, and the glucose concentration was considered to be constant throughout the experiment. Recycling of

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14-C glucose metabolites could result in underestimation of glucose uptake calculated in this manner. That this does not occur is suggested by the similarity between 14-C glucose and 14-C-2-deoxy-glucose uptake.[7] In addition, chromatography of the media after incubation revealed no significant accumulation of nonglucose label.[6]

RESULTS

Effects of maternal fasting. Whereas a sixteen-hour fast, ending seventy-two hours prior to sacrifice (table 1, experiment 1) had no effect on either maternal plasma glucose or insulin concentrations, fasting for the sixteen hours immediately preceding sacrifice (table 1, experiment 2) produced significant reductions in maternal glucose and insulin when compared to twenty-two-day pregnant nonfasted rats (table 1).

Glucose concentration was significantly increased in the fetuses from both groups of fasted mothers. Fetal insulin was unchanged in the fetuses from mothers fasted at seventy-two hours prior to sacrifice; it fell significantly in the fetuses from mothers fasted just prior to sacrifice.

Fasting for sixteen hours, seventy-two hours prior to study did not effect fetal hepatic or myocardial glycogen concentrations (table 2, experiment 1); fasting immediately preceding sacrifice, however, significantly lowered glycogen in both these fetal organs (table 2, experiment 2).

Effects of alloxan diabetes. Intravenous injection of alloxan into the mother produced profound increases in both maternal and fetal glucose (table 3). Whereas maternal insulin concentrations were reduced below detectable levels, fetal insulin concentrations were not significantly altered by maternal alloxan injections. Direct intraperitoneal injection of alloxan into the fetuses had no effect on maternal or fetal glucose or insulin (table 3).

Fetal myocardial glycogen was significantly increased and hepatic glycogen significantly decreased by maternal diabetes (table 4). Fetal alloxan injection was without effect on either hepatic or myocardial glycogen (table 4).

Effects of fasting and alloxan diabetes on fetal myocardial 1-14C-glucose uptake and response to insulin. As can be seen in table 5, neither fasting nor alloxan diabetes altered 1-14C-glucose uptake by the isolated fetal rat heart. Whereas insulin stimulated myocardial glucose uptake, increments were comparable in both experimental and control animals.

DISCUSSION

Fasting. Our findings confirm previous observations that fasting results in a decrease in maternal plasma glucose and insulin concentrations in the pregnant rat.[22] Whereas fetal insulin also decreased following a sixteen-hour fast, fetal glucose actually increased significantly. This increase in glucose was seen in fetuses from mothers fasted for sixteen hours either at three days or immediately prior to sacrifice.

Although increased fetal plasma glucose during fasting in the face of lowered maternal glucose concentrations has been noted by others,[4,18] the mechanisms for this increase have not been deter-

TABLE 1

Effects of fasting on maternal and fetal glucose and insulin concentrations

Fetal Age at Sacrifice	Experimental Conditions	Maternal Glucose (mg./100 ml.)	Maternal Insulin (μU./ml.)	Fetal Glucose (mg./100 ml.)	Fetal Insulin (μU./ml.)
Experiment 1					
21 day	nonfasted	97 ± 6 (4)	62 ± 15 (4)	16 ± 2 (10)	147 ± 7 (2)
21 day	mothers fasted for 16 hrs. 3 days prior to sacrifice, then refed	103 ± 4 (4)	95 ± 10 (4)	26 ± 3 (10)	153 ± 2 (2)
p value*		NS	NS	< 0.02	NS
Experiment 2					
22 day	nonfasted	139 ± 24 (4)	91 ± 21 (4)	35 ± 1 (30)	152 ± 4 (5)
22 day	mothers fasted for 16 hrs. immediately prior to sacrifice	69 ± 7 (4)	23 ± 14 (4)	43 ± 1 (31)	130 ± 4 (7)
p value*		< 0.05	< 0.05	< 0.001	< 0.01

( ) - maternal—number of animals; fetal—number of pooled samples, each pool being obtained from one to four fetuses of the same litter from four mothers.

* - fasted versus nonfasted.

NS - no significant difference.

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TABLE 2

Effects of fasting on fetal hepatic and myocardial glycogen content

Fetal Age at Sacrifice	Experimental Conditions	Hepatic glycogen (μmole/gm. wet wt.)	Myocardial glycogen (μmole/gm. wet wt.)
Experiment 1			
21 day	nonfasted	213 ± 8 (9)	80 ± 3 (17)
21 day	mothers fasted for 16 hrs. 3 days prior to sacrifice, then refed	206 ± 12 (9)	75 ± 3 (20)
p value*		NS	NS
Experiment 2			
22 day	nonfasted	293 ± 11 (24)	96 ± 3 (22)
22 day	mothers fasted for 16 hrs. immediately prior to sacrifice	178 ± 10 (24)	64 ± 6 (24)
p value*		< 0.001	< 0.001

( ) - number of animals.
* - fasted versus nonfasted.
NS - no significant difference.

mined. Diminished fetal hepatic glycogen, also observed during fasting,[4,10] may be relevant to these glucose changes. Conceivably, decreased synthesis and/or increased degradation of hepatic glycogen could account for both the decreased liver glycogen and the increased plasma glucose observed in the fetuses.

Whereas insulin does not play a major role in the moment to moment control of fetal glucose uptake before twenty days' gestation[8] it is important in the regulation of glycogen production in later fetal life.[6,33] At twenty-two days' gestation, the observed decrease in fetal insulin following a sixteen-hour fast may have resulted in diminished glycogen synthesis. Whereas the placental transfer of glucose from mother to fetus is known to occur,[42] the effect of fasting on this process has not been studied. If one assumes that placental transfer of glucose from mother to fetus remains constant during fasting, decreased hepatic glucose uptake and glycogen storage would result in the observed increase in fetal glucose concentrations.

Increased glycogenolysis could also account for both decreased fetal hepatic glycogen and elevated plasma glucose concentrations following a sixteen-hour fast. Glucagon and catecholamines are both potent stimulators of glycogenolysis in the adult rat.[13,35] Although maternal catecholamine secretion is markedly increased with fasting in late pregnancy[21] the effect that this hormone might have on fetal hepatic glycogen is unknown. The fetal pancreas is capable of secreting glucagon,[16] but the ability of this hormone to activate the phosphorylase enzyme system in the fetal animal appears limited.[9,17,36] Indeed the data available suggest that the ability of the fetus to in-

TABLE 3

Effect of alloxan diabetes on fetal and maternal glucose and insulin concentration*

Injection	Site of Injection	Maternal Glucose (mg./100 ml.)	Maternal Insulin (μU./ml.)	Fetal Glucose (mg./100 ml.)	Fetal Insulin (μU./ml.)
Saline	Maternal I.V.	109 ± 5 (4)	90 ± 13 (4)	34 ± 7 (3)	198 ± 18 (3)
Alloxan	Maternal I.V.	791 ± 59 (4)	< .5 (4)	617 ± 50 (3)	179 ± 15 (3)
p value†		< .001	——	< .001	NS
Saline	Fetal I.P.	134 ± 25 (4)	91 ± 21 (4)	27 ± 2 (4)	162 ± 12 (4)
Alloxan	Fetal I.P.	105 ± 7 (8)	82 ± 8 (4)	23 ± 3 (4)	159 ± 7 (4)
p value†		NS	NS	NS	NS

( ) - maternal—number of animals; fetal—number of pooled samples, each pool being obtained from one to four fetuses of the same litter from four mothers.
* - alloxan or saline injection on day 18 of gestation, with sacrifice on day 21.
† - saline versus alloxan.
NS - no significant difference.

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TABLE 4

Effect of alloxan injection on fetal hepatic and myocardial glycogen content

Injection	Site of Injection	Hepatic glycogen (μmole/gm. wet wt.)	Myocardial glycogen (μmole/gm. wet wt.)
Saline	Maternal	264 ± 21 (18)	66 ± 4 (18)
Alloxan	Maternal	203 ± 10 (17)	127 ± 3 (18)
p value*		< 0.02	< 0.001
Saline	Fetal	210 ± (6)	61 ± 8 (8)
Alloxan	Fetal	223 ± 17 (6)	70 ± 5 (10)
p value*		NS	NS

( ) - number of animals.
* - saline versus alloxan.
NS - no significant difference.

itiate hepatic glycogenolysis at all may be significantly limited.[5,33]

We would conclude from the above that decreased hepatic glycogen synthesis secondary to decreased fetal insulin concentrations is the more likely explanation for the observed increased fetal glucose following a fast. Clearly other factors must be operative, for when animals are fasted for sixteen hours three days prior to sacrifice and then refed, fetal blood sugar is elevated despite unchanged glycogen and insulin values. Certainly more studies in this area, as well as in the role of fetal gluconeogenesis during fasting[17] are required to resolve these questions.

As in the liver, fasting also results in diminished glycogen stores in the fetal myocardium. These results have been reported previously[34] and are in contrast to an elevation in adult myocardial glycogen levels after fasting noted by others.[13,32] The balance between glycogen synthesis and breakdown may have been partially responsible for diminished fetal myocardial glycogen stores, i.e. lowered insulin values may have retarded glycogen production while glycogenolysis either increased or remained constant. Other explanations may be offered for the lowered glycogen content in the fetal heart. During fasting the adult myocardium preferentially oxidizes fatty acids over glucose,[29,31] and this may well account for the increased glycogen concentrations noted under these conditions. The fetal myocardium is unable to metabolize fatty acids;[40,43] hence during fasting the fetus in contrast to the adult could not utilize fatty acid substrates to maintain constant glycogen storage.

Diabetes. In previous investigations diabetes has been induced either prior to conception[24,25,41] or prior to the twelfth day of pregnancy[8,11,26,27] before the fetal pancreas has developed.[15] Whereas in the previous studies fetal insulin concentrations were increased by maternal diabetes, in the present studies they were unchanged by maternal alloxan diabetes. We conclude that maternal diabetes must be of greater duration than seventy-two hours in order to produce fetal hyperinsulinism.

Fetal insulin was unaffected by alloxan administration directly into the fetus. Others have shown that

TABLE 5

Effect of fasting and alloxan diabetes on fetal myocardial glucose uptake

Treatment	Age	Basal uptake (μmole/gm. wet wt.)	Insulin stimulated uptake (5,000 μU./ml.)	p value*
Experiment 1				
Control	21	12.5 ± 0.8 (14)	15.7 ± 0.9 (15)	< 0.02
Fasted	21	14.2 ± 0.9 (16)	16.4 ± 0.6 (16)	< 0.05
Experiment 2				
Control	21	8.9 ± 0.8 (4)	17.3 ± 1.0 (4)	< 0.001
Diabetic	21	10.2 ± 0.6 (3)	17.1 ± 2.5 (4)	< 0.05
Experiment 3				
Control	22	5.0 ± 0.9 (6)	8.3 ± 1.1 (7)	< 0.05
Diabetic	22	5.2 ± 0.8 (5)	10.0 ± 0.9 (6)	< 0.02

( ) - number of animals.
* - insulin stimulated versus basal.
In all cases the difference between the uptake in the control and experimental animals is not significant.

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the fetal and neonatal rat are resistant to the diabetogenic effect of alloxan.[2,37,39] Although this could mean that alloxan has no effect on the fetal β-cell or is unable to penetrate the placental membranes, it is also possible that the destroyed fetal β-cells are replaced by newly differentiated cells; β-cell replication continues after birth in the rat.[15]

Whereas at twenty-two days of gestation an increase in hepatic glycogen has been observed in diabetic animals,[20,28] hepatic glycogen content of the twenty-one-day diabetic fetuses in our studies was decreased. We believe this difference in hepatic glycogen can be explained by this age difference. The ability of the fetal rat to regulate glucose phosphorylation is severely limited. Glucokinase does not develop until two weeks after birth.[39] Glucose-6-phosphatase develops late in the twenty-first or early in the twenty-second day of gestation.[5] Although this latter enzyme operates in the direction of glucose in the adult, it appears to play a major role in glucose phosphorylation in the fetus.[14] We may speculate that as more glucose-6-phosphate is made available by the increased glucose-6-phosphatase activity, more is incorporated into glycogen. In any case, it appears that prior to day 22 of gestation diabetes does not increase fetal hepatic glycogen in spite of remarkably high plasma glucose concentrations, perhaps because of limited hepatic ability to phosphorylate glucose.

Maternal diabetes induced on day 18 resulted in an increase in fetal myocardial glycogen on day 21. For reasons previously discussed, these changes could not have resulted from increased myocardial fatty acid oxidation as is the case in the adult rat.[31] Other explanations may be offered. Previous studies have demonstrated increased incorporation of C-14 glucose into glycogen in the twenty-one-day fetus exposed to maternal hyperglycemia.[8] Furthermore, the fetal myocardium is capable of increased glycogen formation from 1-14-C glucose.[6] In the presence of adequate insulin, then, the observed fetal hyperglycemia may well have augmented glucose uptake and incorporation into myocardial glycogen. Certainly regulation of fetal myocardial glycogen metabolism is not well understood, and more studies in this area need to be performed.

Both fasting and diabetes reduce basal and insulin-stimulated glucose uptake in the adult rat.[28,30] This results from preferential fatty acid oxidation[29] in these animals and, in diabetes, decreased ability to phosphorylate glucose.[28] Fetal myocardial glucose uptake was unaltered by either maternal fasting or diabetes mellitus. These results were anticipated since the fetus lacks the ability to oxidize fatty acids and the insulin deficiency necessary to produce decreased glucose phosphorylation was not observed in the fetus.

CONCLUSION

From the studies performed, it is concluded that whereas the twenty-one- or twenty-two-day fetus has some ability to adjust to maternal fasting and diabetes, such manipulations of the mother can cause significant alterations in fetal carbohydrate metabolism. Additional data will be required to clarify the mechanisms responsible for these observed changes; such studies will require meticulous attention to fetal age and maternal nutrition.

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