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Studies on Gluconeogenesis and Ketogenesis in Isolated Hepatocytes from Alloxan Diabetic Rats[1] (39125)

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The induction of experimental diabetes in rats causes several alterations in liver. These include increases in glucose production (1–5), ketogenesis (6–8), and decrease in protein synthesis (9, 10). Previously these studies have been carried out using intact animals, liver slices, and perfused liver. The present studies were undertaken to determine gluconeogenesis and ketogenesis by isolated hepatocytes obtained from alloxan diabetic rats.

Materials and methods. Animals. Male, Cox rats weighing from 150–180 g were used. They were fed *ad libitum* on Purina Laboratory Chow. Alloxan diabetes was produced by intravenous injections of alloxan monohydrate (40 mg/kg of body weight) in the manner described previously (3). Rats exhibiting blood glucose levels greater than 300 mg% five days following alloxan administration were started on insulin and maintained on 4 units protamin zinc insulin daily (Lilly) for 2 weeks prior to use. These diabetic rats were used at various time intervals after the withdrawal of protamin zinc insulin (0, 48, 72, 96 hr). Both diabetic and normal rats weighing from 180–220 g were used for all the studies reported here.

Preparation of hepatocytes. Normal and diabetic rats were anesthetized with Na-pentobarbital and the liver was rapidly removed and placed in a Miller perfusion apparatus, perfused for 15 min with 100 ml of Hanks calcium-free buffer containing 1.5% albumin (Sigma Fraction V) and 10 mg each of streptomycin and penicillin G. Following this initial perfusion, collagenase (20 mg, Sigma Type I, 130 units/mg) was added, and perfusion was continued for 10–15 min. The liver was removed, finely minced, and bubbled gently with 95% O_2 and 5% CO_2 for 1 min. Cells were isolated as described previously (11, 12) and were brought to a final volume of 30–40 ml. Isolated hepatocytes were used immediately for all metabolic studies.

Incubation of the hepatocytes. A 1 ml aliquot of the final cell suspension (55–75 mg) was incubated in 3 ml of Umbreit Ringer bicarbonate buffer (25 m*M*) in stoppered 1-oz plastic vials (Nalgene 2002) with various substrates. The vials were gassed with 95% O_2 and 5% CO_2 for 5 min, and cells were incubated for 1 hr. At the end of incubation the vials' contents were placed in ice cold conical centrifuge tubes and were centrifuged at 2000 rpm in an International centrifuge for 10 min. The supernatant was assayed for glucose. All experiments were conducted in duplicate, and all values reported here are the mean ± the standard error of the mean.

Analytical methods. Glycogen determination was carried out by the method of Good *et al.* (13). After the precipitation it was washed and hydrolyzed, and glucose was assayed by the glucose oxidase method (14). Blood glucose was determined as described previously (3). Lipid content was estimated by weighing free fatty acids isolated from ethanol KOH supernatant obtained after glycogen (15) precipitation. The supernatant was poured into ground stoppered tubes containing 1 ml of concentrated HCl. Heptane (3.0 ml) was added, mixed vigorously, and the upper heptane layer was removed. This procedure was repeated twice. Heptane extracts were pooled and washed with distilled water, evaporated to dryness, and isolated fatty acids were then weighed. Results are calculated as mg lipid/g of cells of liver. Ketone bodies were measured by the fluorometric method (16). Radioactive glucose

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TABLE I. LIVER GLYCOGEN CONTENT AND BLOOD LEVELS OF NORMAL RATS AND DIABETIC RATS WITHDRAWN FROM INSULIN FOR VARIOUS PERIODS OF TIME[a]

			Diabetic (hours after last insulin injection)			
	Normal fed	18–24-hr-fasted	0	48	72	96
	Glycogen (μmoles of glucose equivalents/g wet wt)					
Whole liver	230 ± 46 (4)	Trace	803 ± 53 (3)	760 ± 120 (3)	245 ± 80 (5)	203 ± 82 (6)
Isolated cells	105 ± 14 (4)	Trace	710 ± 55 (3)	582 ± 61 (3)	65 ± 25 (5)	106 ± 22 (5)
	Blood glucose (mg glucose/100 ml of blood)					
	110 ± 10 (4)	80 ± 8 (4)	70 ± 9.4 (4)	296 ± 15 (4)	322 ± 8.0 (4)	350 ± 31 (4)

[a] Values are mean ± SEM of (*N*) observations.

TABLE II. TOTAL LIPID CONTENT OF LIVERS FROM DIABETIC RATS[a]

	Hours after last insulin injection			
	0	48	72	96
Whole liver	45.4 ± 9.6	43.9 ± 5.4	62.1 ± 3.4	42.4 ± 0.5
Isolated liver cells	33.2 ± 3.1	34.3 ± 3.0	53.3 ± 2.1	31.9 ± 2.6

[a] Lipid was isolated as described in text. Values are mean ± SEM of four observations and are expressed as mg lipid/g wet wt.

was isolated as phenyl osazone as described previously (3).

Results. Studies on glycogen, lipid content, and blood glucose levels in normal and diabetic rats. Glycogen levels in whole liver and isolated cells from alloxan diabetic rats withdrawn from insulin for various periods of time are summarized in Table I. It can be seen from this table that insulin treatment maintained near-normal blood glucose levels and caused an increase in glycogen deposition. Two days after the last injection, blood glucose levels were elevated. Glycogen levels fell below normal by the third day. The third day after the last injection the rats displayed a diabetic syndrome marked by progressive hyperglycemia and glycogen depletion. Table II summarized lipid content of whole liver and isolated liver cells obtained from normal and diabetic rats. It is evident from this table that there is a significant increase in lipid content in liver at 72 hr after the insulin withdrawal.

Studies on glucose production. Net glucose production by isolated hepatocytes progressively increased with time up to 72 hr after the last *in vivo* insulin injection (Table III). Glucose production decreased at 96 hr. The same pattern was observed with the incorporation of labeled bicarbonate into glucose (Table IV), indicating that the increase in glucose production was due to an increased flow of carbon from noncarbohydrate sources into glucose. Glucose production at 48 hr after termination of insulin treatment was similar to that observed in cells obtained from 18–24-hr-fasted rats and three to 30 times that observed in cells from normally fed rats. Maximal glucose production was observed at 72 hr following the withdrawal of insulin with 10 m*M* gluconeogenic precursors. Increasing the concentration of lactate or fructose above 10 m*M* did not further increase glucose production at any time after insulin withdrawal. These results agree with the findings of Wagle and Ashmore (3) using liver slices and with those of Jefferson *et al.* (17) using perfused liver, that hepatic glucose production is elevated in diabetes.

Studies on ketogenesis. Ketogenesis in hepatocytes from diabetic rats, like glucose production, peaked at 72 hr after the last insulin injection (Table V). A 10-fold increase in ketogenesis was paralleled by an increase in hepatic lipid content in diabetic rats (Table II). This suggests that the increase in hepatic ketogenesis may be due to the increased availability of fat for oxidation. The increase in hepatic lipid content and

TABLE III. NET GLUCOSE PRODUCTION BY ISOLATED LIVER CELLS FROM NORMAL AND DIABETIC RATS[a]

Substrate (10 m*M*)	Normal fed rat	Normal fasted rat	Diabetic rats (hours after last insulin injection) 0	48	72	96
No substrate	49.9 ± 5.8 (5)	2.3 ± 0.6	58.6 ± 3.9 (6)	10.5 ± 3.5 (5)	6.6 ± 1.0 (8)	3.4 ± 0.9 (5)
Net glucose production						
Alanine	2.3 ± 0.2 (4)	18.2 ± 1.2	1.7 ± 0.9 (6)	10.8 ± 3.2 (4)	53.3 ± 9.0 (6)	34.4 ± 6.4 (5)
Lactate	9.8 ± 2.9 (4)	42.5 ± 7.0	21.2 ± 1.8 (6)	37.8 ± 3.9 (5)	91.3 ± 14.0 (6)	48.0 ± 7.5 (5)
Pyruvate	4.3 ± 1.8 (4)	35.2 ± 3.2	11.2 ± 1.4 (6)	16.9 ± 2.7 (4)	61.0 ± 7.8 (6)	41.6 ± 7.7 (5)
Fructose	54.6 ± 5.3 (4)	76.1 ± 6.7	57.3 ± 3.8 (6)	105 ± 12.0 (4)	162 ± 22 (6)	143 ± 32 (5)

[a] Isolated liver cells were incubated in the presence or absence of substrate for 1 hr. Net glucose production was determined by subtracting engenous production (no substrate) from glucose production in the presence of substrate. Values are mean ± SEM of (*N*) observations and are expressed as μmoles glucose/g wet wt/hr.

TABLE IV. INCORPORATION OF [^{14}C]$NaHCO_3$ INTO GLUCOSE BY ISOLATED LIVER CELLS FROM NORMAL AND DIABETIC RATS[a]

Substrate (10 m*M*)	Normal fed rats	Hours after last insulin injection 0	48	72	96
No substrate	1.5 ± 0.22 (4)	2.8 ± 0.23 (5)	1.1 ± 0.08 (5)	1.9 ± 0.26 (6)	0.42 ± 0.07 (4)
Alanine	1.4 ± 0.01 (4)	2.3 ± 0.24 (6)	4.6 ± 0.27 (6)	7.5 ± 0.11 (6)	5.90 ± 0.61 (5)
Lactate	3.0 ± 0.5 (4)	4.1 ± 0.54 (6)	9.3 ± 0.1 (6)	21.0 ± 4.1 (6)	8.50 ± 0.74 (5)
Pyruvate	6.3 ± 0.39 (4)	10.0 ± 0.49 (5)	9.4 ± 0.94 (6)	17.5 ± 2.1 (6)	13.9 ± 1.4 (5)

[a] Isolated liver cells were incubated for 1 hr in the presence of various substrates and 0.5 μCi of [^{14}C]$NaHCO_3$. Values are mean ± SEM of (*N*) observations and are expressed as μmoles of CO_2 incorporated into glucose/g wet wt/hr.

TABLE V. KETONE BODY FORMATION IN ISOLATED LIVER CELLS FROM DIABETIC RATS[a]

	Hours after last insulin injection			
	0	48	72	96
No substrate	3.8 ± 0.7	12.2 ± 4.4	37.9 ± 4.1	23.4 ± 0.9
Fructose (10 mM)	5.9 ± 0.5	9.3 ± 1.3	17.1 ± 2.4	14.9 ± 1.5
Lactate (10 mM)	3.6 ± 0.9	4.0 ± 1.3	10.4 ± 1.2	9.8 ± 2.4

[a] Isolated liver cells from diabetic rats withdrawn from insulin for various time periods were incubated in the presence or absence of substrate for 1 hr. Values are mean ± SEM of four observations and are expressed as μmoles ketones/g/hr.

ketogenesis in diabetics is in agreement with previously reported observations (5–8). The addition of either 10 m*M* lactate or fructose suppressed ketone body formation by 50 to 70% in liver cells isolated from diabetic rats deprived of exogenous insulin for 48, 72, or 96 hr. However, these substrates had no effect on ketone body production in liver cells isolated from control rats (0 hr). Both lactate and fructose have antiketogenic effects not only in perfused liver from fasted rats (18) but also in isolated hepatocytes from diabetic rats as observed in the present studies.

Discussion. The increase in hepatic glucose production in experimental diabetes is well documented (1–5) and has been reconfirmed in these studies by using isolated hepatocytes. It has been generally assumed that metabolic alterations that occur in the diabetic liver are due to changes in metabolic pathways in parenchymal cells. The studies presented here confirm that assumption. The massive changes in glucose production and in other parameters studied here with isolated hepatocytes in experimental diabetes indicate that hepatocytes, and not other liver cell types, are responsible for the metabolic alterations observed *in vivo* or with other *in vitro* preparations.

Hepatic gluconeogenic enzymes increase in diabetes (1, 19). Pyruvate carboxylase (PC) and phosphoenol pyruvate carboxy kinase (PEPCK) increase three to sevenfold while fructose-1,6-diphosphatase and glucose-6-phosphatase increase only two to threefold. The increases in glucose production observed in diabetic rats over controls using alanine (14–34-fold), lactate (sevenfold), or pyruvate (sixfold) were greater than that observed when fructose (two to threefold) was used as a gluconeogenic substrate. This supports the role of alanine in gluconeogenesis as suggested by Felig *et al.* (20). Since lactate, pyruvate, or alanine enter through PC and PEPCK, and fructose enters through triose phosphate and is controlled by fructose-1,6-diphosphatase and glucose-6-phosphatase, these increases in glucose production correlate with the changes in the respective enzyme levels (1, 19). However, the increase in glucose production in diabetes may not be entirely due to increases in gluconeogenic enzymes; since amino acid incorporation into protein decreases (3), endogenous amino acids may be incorporated into glucose. This is in agreement with the observation that the incorporation of exogenously administered ^{14}C-amino acids into glucose increases in diabetes (3).

Recently it has been shown that the high rates of glucose production from gluconeogenic substrates are dependent upon fatty acid oxidation (21, 22). The observation that increases in hepatic lipid content and ketogenesis paralleled the increase in glucose production in isolated liver cells strongly suggests that the increase in glucose production in experimental diabetes may be, in part, due to increased fatty acid oxidation.

The studies presented here indicate that liver cells are sensitive to hormones and should be useful in elucidating mechanisms of hormone interactions. The use of isolated hepatocytes in metabolic studies may take

on even more importance in light of the development of perfused systems for pancreatic islet cells (23) and fat cells (24). There is no reason to suspect that isolated liver cells cannot be used in such a system, alone or in combination with cells isolated from other tissues. With such systems the interactions between various tissues may be studied *in vitro*, as we have also observed that these isolated hepatocytes respond to various hormones at physiological concentrations (25–29).

Summary. Gluconeogenesis and ketogenesis were studied in isolated hepatocytes obtained from normal and alloxan diabetic rats. Insulin treatment maintained near-normal blood glucose levels and caused an increase in glycogen deposition. The third day after insulin withdrawal the rats displayed a diabetic syndrome marked by progressive hyperglycemia and glycogen depletion. Net glucose production in liver cells isolated from alloxan diabetic rats progressively increased with time up to 72 hr after the last *in vivo* insulin injection. Maximal glucose production was observed at 72 hr with 10 m*M* alanine, lactate, pyruvate, or fructose. Glucose production decreased at 96 hr. The same pattern was observed with the incorporation of labeled bicarbonate into glucose. Ketogenesis in liver cells and hepatic lipid content also peaked at 72 hr.

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