Studies of the development of diabetic ketosis in the rat

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Abstract Plasma glucose, free fatty acid, ketone, and triglyceride concentrations were measured at frequent intervals after the administration of alloxan to rats. Hepatic triglyceride levels were determined in the same animals. During the second 24-hr period after alloxan administration, severe ketoacidosis developed and triglyceride concentrations in the liver became markedly elevated. This finding was incompatible with the thesis that enhanced ketogenesis under circumstances of increased free fatty acid delivery to the liver requires diminished triglyceride synthesis.

Plasma insulin and glucagon concentrations were determined at each time point. Initial changes in plasma glucose, ketones, free fatty acids, and triglycerides were accompanied by a fall in insulin concentrations, but no change occurred in glucagon levels. However, concentrations of the latter hormone increased dramatically in the second 24 hr after alloxan treatment and probably contributed to the development of the extreme hyperglycemia observed during this time period.

Supplementary key words alloxan · diabetes · glucagon · insulin · ketogenesis · triglycerides

THE INTERRELATIONSHIP between abnormalities in lipid metabolism and the production of ketone bodies by the liver has received extensive study. It is now clear that a simple increase in delivery of free fatty acids from peripheral fat stores to the liver is not sufficient in itself to induce ketone production and that primary hepatic regulatory factors for the control of ketogenesis must exist (1–5). Considerable evidence has accrued to suggest that such regulation resides, at least in part, in the capacity of hepatic tissue to utilize incoming fatty acids for triglyceride synthesis; i.e., insofar as peripheral fatty acids are rapidly utilized for esterification they would be unavailable to enter the β-oxidation pathway for the ultimate formation of ketone bodies (4–6). In this regard it can be shown that triglyceride synthesis from oleic acid is diminished in starvation ketosis and restored toward normal when ketosis is reversed by glycerol and other antiketogenic agents (5, 6). On the other hand, it has long been known that the liver in diabetic animals has increased triglyceride concentrations (7–9), an observation which on the face of it would appear incompatible with the thesis that diminished triglyceride synthesis is necessary for enhanced ketone body formation. While hepatic and blood lipid values have been reported in diabetes induced by pancreatectomy, antiinsulin antibodies, and β-cell destructive agents, it seemed important to investigate the temporal relationship of changes occurring in the various lipid components and plasma ketone bodies during the induction of diabetic ketosis. Of critical interest was the relationship between triglyceride accumulation in the liver and the onset of ketogenesis. In addition, we have measured plasma insulin and glucagon concentrations at each time point in the sequence.

MATERIALS AND METHODS

Treatment of animals

All experiments were carried out utilizing male Sprague-Dawley rats weighing 120–140 g. The animals were maintained on a fat-free diet containing 58.5% sucrose, 21% casein, and the necessary vitamins and minerals (General Biochemicals, Chagrin Falls, Ohio). Groups of animals (40–60) were given alloxan mono-

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hydrate intravenously in a dose of 60 mg/kg of body weight. The animals were subsequently allowed free access to food and water until killed, except for one set of experiments in which the rats were fasted after injection of the drug. At each time point, animals were anesthetized with pentobarbital, the abdomen was opened, and 3–5 ml of blood was drawn from the aorta. The liver was then immediately removed and frozen in liquid nitrogen.

Assays on blood

Blood was collected in tubes containing EDTA-Trasylol to allow for hormone assays (10). After separation of the plasma, acetoacetate and β-hydroxybutyrate were assayed enzymatically (11). Nonesterified fatty acids were determined by titration as described by Trout, Estes, and Friedberg (12). Plasma triglycerides were measured by the method of Van Handel and Zilversmit (13). After their separation from the triglyceride fraction, phospholipids were quantitated by measurement of inorganic phosphorus released upon alkaline hydrolysis (14). Glucose concentrations were measured by the glucose oxidase method (15). Aliquots of plasma from two to four animals were pooled for determination of insulin and glucagon by radioimmunoassay (10, 16). The latter test utilized an antibody specific for pancreatic glucagon.

Assays on liver

Frozen livers were extracted in a blender with chloroform–methanol 2:1 (v/v) in a ratio of 40 ml/g of tissue. Triglyceride and phospholipid fractions were separated on silicic acid columns (13) and assayed as described above.

Fatty acid and cholesterol synthesis

Synthesis of fatty acid and cholesterol was measured in liver slices utilizing [2-^{14}C]acetate as substrate as previously described (17).

RESULTS

The sequence of events taking place during the onset of acute diabetic ketoacidosis was studied in animals that were allowed free access to food and in those that were fasted. As indicated in Fig. 1, very significant weight loss, approaching 20%, occurred during the 48 hr after administration of alloxan. The decrease was continuous in the fasted animals, but in the fed animals the initial weight loss was followed by weight gain between 12 and 24 hr, after which a rapid decline was noted. At 48 hr, no significant difference existed between the two groups.

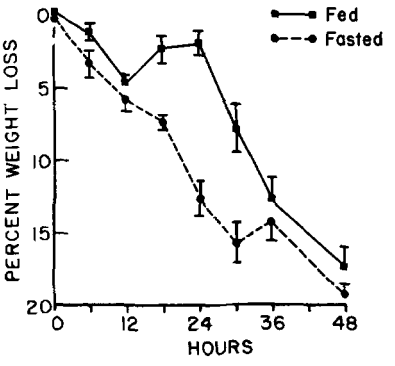

FIG. 1. Weight loss after administration of alloxan. Each point (mean ± SEM) represents percentage change from initial weight for 12–18 animals in the fed group and 6 animals in the fasted group.

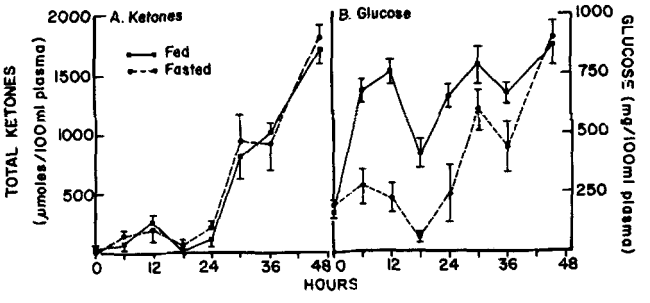

FIG. 2. Changes in plasma ketone and glucose concentrations after the administration of alloxan. Results are given as means ± SEM for the number of animals shown in Fig. 1.

Plasma glucose and ketones

In the fed animals, plasma glucose levels were markedly elevated by the 6-hr time point, with a mean value of 690 mg/100 ml, as shown in Fig. 2*B*. In contrast, the fasted rats had a mean glucose concentration of only 275 mg/100 ml, a value definitely elevated above normal fasting values but not distinctly different from the initial postprandial level. The wide difference between the two groups reflects the relative contributions of hepatic glucose release and dietary carbohydrate at this early time point. By 48 hr, similar values were found in both fed and fasted groups, suggesting a predominant role for endogenous glucose production in each case. The second point of interest is the pronounced drop in plasma glucose concentration occurring at 18 hr in both sets of animals.[1] A second fall appears to occur at 36 hr, but it is not statistically significant. Similar patterns of glucose response to β-cytotoxic agents have been previously described (18, 19).

[1] The relative hypoglycemia found at 18 hr can be very severe. Several animals in the fasted group died with hypoglycemic convulsions at this time point.

Total ketone concentrations in the plasma (Fig. 2*A*) were not affected by dietary intake. Slight elevations, approaching values seen in fasting ketosis, were observed at 12 hr, but the concentrations returned to near normal levels at 18 hr. Brisk acceleration of ketogenesis reappeared by 30 hr, and at 48 hr concentrations of 17–18 mM were reached.

Plasma free fatty acids, triglycerides, and phospholipids

Plasma free fatty acids exhibited changes remarkably similar to those seen with plasma glucose, as shown in Fig. 3. Concentrations more than doubled by 12 hr and returned to near baseline levels at 18 hr. A secondary rise to peak values was seen in the second 24 hr, at which time absolute levels were higher in the fed group.

Changes in triglyceride levels paralleled those of free fatty acids, with an early rise followed by a return to normal values at 18 hr. At 30 hr, only about half the animals in the fed group had lipemic plasma (resulting in a very large standard error of the mean), while at 36 hr, all the values were elevated. Lipemia was also noted in the fasted group, but it was not always present. The higher values seen in the fed animals were not due to ingested triglyceride, since the animals were fed a fat-free diet. Presumably, the exogenous carbohydrate, which resulted in higher blood glucose levels, was responsible for the effect, although the mechanism is uncertain. While carbohydrate loading can stimulate hepatic triglyceride synthesis (20), the effect could also be exerted on peripheral disposal of the neutral lipid (21). It should be noted, although the data are not shown, that plasma phospholipids were also measured and showed changes which were qualitatively similar to those of the triglycerides, though the maximum increase in absolute terms was considerably less.

Plasma insulin and glucagon

Sequential changes in plasma insulin and glucagon concentrations after administration of alloxan are shown in Fig. 4. As has been noted previously (19), insulin values fell initially, only to rise sharply at 18 hr. The rise in insulin, which was not affected by fasting, was temporally associated with the decrease in plasma glucose, ketones, free fatty acids, and triglycerides described above. Plasma immunoreactive glucagon values, on the other hand, were unchanged until 24–30 hr after the injection of alloxan. At the 48 hr time point, both fed and fasted rats had glucagon concentrations some 10–15 times greater than control values.

Hepatic triglycerides and phospholipids

Alterations in hepatic triglyceride content during the development of diabetic ketoacidosis are shown in Fig. 5. Initial increases in mean neutral fat content were observed in the fed animals by 30 hr, and at the 36 hr point, concentrations were approximately five times greater than values obtained at time zero. Accumulation

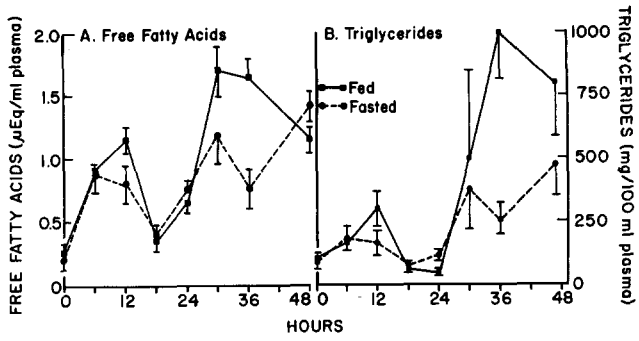

FIG. 3. Changes in plasma free fatty acid and triglyceride concentrations after administration of alloxan. Results are given as means ± SEM for the number of animals shown in Fig. 1.

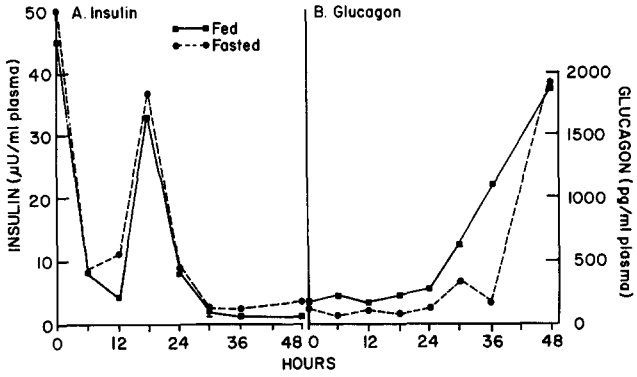

FIG. 4. Changes in plasma insulin and glucagon concentrations after administration of alloxan. As indicated in the text, each hormone was measured in a pooled sample of from two to four animals. Since only two or three values were obtained at each time point, SEM were omitted.

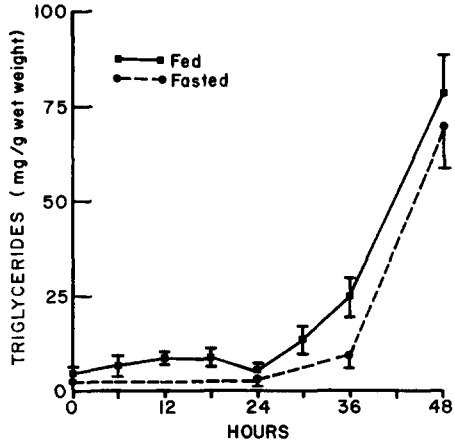

FIG. 5. Changes in hepatic triglyceride concentrations after administration of alloxan. Results are given as means ± SEM for the number of animals shown in Fig. 1.

continued through 48 hr, when a mean value of 79 mg/g of liver was obtained. While early time points were not examined in the fasted rats, it appears that a similar pattern developed, since no increase was found at 24 hr but by 48 hr a concentration of 69 mg/g was obtained. No statistically significant changes were seen in phospholipid content throughout the 48-hr period (values not shown). Similar findings were previously reported in diabetes induced by pancreatectomy (7).

Hepatic fatty acid and cholesterol synthesis

Fatty acid synthesis has long been known to be decreased in diabetes. While earlier studies in fasted rats suggested that a restoration of lipogenesis to normal did not occur rapidly after feeding (17), we were interested to see if the secondary rise in insulin concentration at 18 hr, which was sufficient to reverse the major plasma abnormalities resulting from alloxan administration, would have an effect on fatty acid synthesis by the liver. The data shown in Fig. 6 indicate that a rise in fatty acid synthesis took place between 12 and 18 hr. However, individual variation was sufficient to render the significance of the results questionable. In the same studies no obvious change in cholesterol synthesis in liver slices was noted.

DISCUSSION

The primary purpose of the present investigation was to examine the relationship of triglyceride accumulation in the liver to the onset of severe diabetic ketoacidosis. This relationship was considered to be of importance in view of the likelihood that competition between the β-oxidative and triglyceride-synthesizing pathways for incoming fatty acids plays a major role in the regulation of hepatic ketogenesis (5, 22). In view of the fact that the ketosis of fasting is associated with diminished triglyceride synthesis in the liver (5, 6, 20) and that the reversal of ketosis with antiketogenic agents such as glycerol and lactate is accompanied by an increased esterification of fatty acids (5, 23), emphasis has been placed on the possibility that the hepatic capacity for triglyceride synthesis is the key factor in determining whether or not ketone body formation is accelerated. According to this thesis, an increased transport of free fatty acids to the liver from peripheral fat stores would result in enhanced ketogenesis only if the fatty acids could not be utilized for triglyceride synthesis. Under this formulation the oxidative pathway is considered to have a capacity which is fixed and large, and the generation of acetyl CoA would be considered limited only by the availability of the substrate fatty acid. The data of the present paper indicate that this viewpoint may be oversimplified or incorrect. Such a conclusion is based primarily on the fact that plasma ketone concentrations in the fed animals increased from 130 ± 42 μmoles/100 ml at 24 hr to 815 ± 143 μmoles/100 ml at 30 hr, a sixfold change. In the same time period, hepatic triglycerides also increased, but only from 8.2 ± 0.78 to 14.0 ± 2.92 mg/g of liver. Subsequently, however, triglyceride concentration increased sharply as ketone concentrations continued to rise. We have assumed that the triglyceride accumulation reflects in large part increased hepatic synthesis in the liver. While diminished hepatic release of triglycerides has been shown in isolated perfused livers from alloxan diabetic rats (24), the marked rise in plasma triglycerides accompanying hepatic fat storage described here suggests that impaired release does not play a major role in vivo.[2] If this interpretation is correct, it is clear that diabetic ketoacidosis develops under circumstances in which triglyceride synthesis is not impaired, but accelerated, and that increased rates of ketogenesis precede the onset of accentuated triglyceride formation.[3] Such a sequence of events does not fit the simple concept that rapid ketogenesis follows only after depression of the triglyceride synthesizing mechanism. At least two possibilities exist to explain the data. The first would be that the mechanisms of ketosis in fasting and in diabetes are different. In the former, a depression of triglyceride synthesis might be primary, while in the diabetic state no such impairment exists.

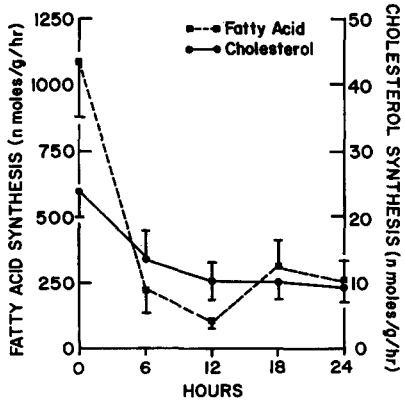

FIG. 6. Changes in hepatic fatty acid and cholesterol synthesis after administration of alloxan in fed rats. Results are given as the means ± SEM for 8–12 animals at each time point.

[2] An alternative to this interpretation could be constructed by assuming that the lipemic plasma resulted primarily from diminished peripheral utilization of triglycerides (21). Should this be true, diminished release of triglycerides from the liver as lipoprotein might be present despite elevated neutral fat levels in the plasma. Under these circumstances, hepatic accumulation of fat might occur with normal or diminished triglyceride synthesis. We consider this sequence possible, but unlikely.

[3] Indeed, several animals at the 30- and 36-hr time points exhibited extremely high ketone concentrations without concomitant elevation in liver triglycerides.

Presumably, in the latter case free fatty acid mobilization would be sufficiently great to supply substrate for both pathways. An alternative possibility would be that, in contrast to current concepts, the rate of triglyceride synthesis is dependent upon the rate of fatty acid flux into the oxidative machinery and not vice versa. According to this view, triglyceride formation would be favored whenever fatty acid oxidation is limited, as in the fed state, or when the oxidative pathway is saturated because of greatly increased fatty acid loads, as in severe diabetic ketoacidosis. The idea that rates of fatty acid oxidation regulate rates of triglyceride synthesis is not without precedent, since similar conclusions were reached by Wittels and Bressler (25) and Wittels and Spann (26) in studies of myocardial fatty acid metabolism. If the capacity for fatty acid oxidation is the limiting factor in the disposal of fatty acids delivered to the liver in fed animals, the point of regulation would probably exist prior to the entry of the fatty acids into the oxidative reactions, since octanoic acid, which bypasses the carnitine transfer mechanism, is oxidized at the same rate in livers from fed and fasted rats (4).

As previously noted by other investigators (19, 27), a clear-cut, three-phase response in blood glucose concentration followed the administration of the β-cytotoxic agent. The lowering of the blood glucose level at 18 hr correlated well with the increased insulin release seen at this time point. The present studies indicate that a similar phenomenon is observed with plasma ketones, free fatty acids,[4] and triglycerides, all of which began to rise as the plasma insulin level fell, reverted toward normal with insulin release, and rose again to peak levels as insulin concentrations decreased once more.

It is of considerable interest that the early events of the alloxan diabetic state are unaccompanied by changes in glucagon concentrations as measured by an immunoassay specific for the pancreatic hormone, while in the second 24-hr period values rise to exceedingly high levels. Similarly high values have been observed in humans during severe diabetic ketoacidosis (10). Considerable attention has been paid to the role played by glucagon in the pathophysiology of diabetes (10, 28). The present data suggest that the initial hyperglycemia, mobilization of free fatty acids, and onset of ketosis occur independently of changes in glucagon concentration and that they are the primary result of insulin deficiency. While glucagon has been postulated to play a role in the generation of ketosis by activating hepatic lipases (29, 30), such an activity would appear to be of little importance in vivo, since peak triglyceride storage occurs in the liver at the very time that glucagon concentrations are highest. In this context it is of interest that pancreatectomized rats display characteristics remarkably similar to those described in the present studies with the exception that blood glucose concentrations after pancreatectomy were less than half those observed here (7). Presumably, the pancreatectomized animals were deficient in pancreatic glucagon. It seems likely, therefore, that the excessive hyperglycemia of the alloxan-treated rats during the second day was the consequence of the grossly elevated glucagon levels. It also seems likely that the glucagon effect is due totally or in part to an acceleration of gluconeogenesis, a conclusion which follows from the observation that the considerable differences in plasma glucose concentrations which exist between fed and fasted animals at an early time point disappear in the second day, when very high values are obtained even in the fasted group.

[4] In data not shown, free fatty acid concentrations were plotted against plasma ketone levels in individual animals. An excellent correlation between these two parameters was observed for the first 18 hr after alloxan treatment in both fed and fasted groups. Thereafter, however, this correlation completely disappeared.

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