Hepatic Autophagy in Uncontrolled Experimental Diabetes and Its Relationships to Insulin and Glucagon

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ABSTRACT Exogenous glucagon is known to increase hepatic lysosomes, but the relationships between endogenous glucagon and insulin levels and hepatic lysosomes have not been examined. To determine if the hormones of the pancreatic islets influence the development of these organelles glycogenosomes, dense bodies, and autophagosomes were morphometrically quantitated in normal rats, in rats with mild streptozotocin diabetes with normal hormone levels, and in rats with severe streptozotocin diabetes with hyperglucagonemia, hypoinsulinemia, and clinical evidence of uncontrolled diabetes and ketoacidosis. In the latter volume density of lysosomes averaged 222.8×10^{-4} (SEM $\pm 19.8 \times 10^{-4}$), significantly above the control value of 75×10^{-4} (SEM $\pm 7.0 \times 10^{-4}$) ($P < 0.0005$); glycogenosomes were absent in the diabetics, the increase being largely the result of increased autophagosomes. Insulin treatment corrected the hyperglucagonemia, hypoinsulinemia, and other manifestations of uncontrolled diabetes and reduced the volume density of lysosomes to 37.4×10^{-4} (SEM $\pm 2.0 \times 10^{-4}$), significantly below both the untreated diabetic rats and the nondiabetic controls ($P < 0.0025$). In mild streptozotocin diabetes, in which hyperglucagonemia, hypoinsulinemia, and other evidence of uncontrolled diabetes were absent, lysosomes averaged 77.6×10^{-4} (SEM $\pm 5.5 \times 10^{-4}$), not different from the controls. A statistically significant correlation between all measurements of lysosomal volume density and plasma glucagon was observed ($r = 0.79$; $P < 0.001$). It is concluded that uncontrolled streptozotocin diabetes in rats is accompanied by hepatic autophagy which may be related to the increased plasma glucagon level and/or the decreased insulin and which is corrected by insulin therapy.

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INTRODUCTION

Ashford and Porter first reported an increase in lysosomes in isolated rat livers perfused with glucagon (1). These findings have been confirmed in vivo by Deter and De Duve (2), Arstila and Trump (3), and Guder, Hepp, and Wieland (4) in studies demonstrating increased hepatic autophagocytosis after intraperitoneal administration of glucagon in intact rats. Although in all of the above studies the concentrations of glucagon were undoubtedly far above the highest levels of endogenous glucagon that occur physiologically or pathophysiologically, they, nevertheless, raised the possibility that this "catabolic" hormone mediates increased formation of these specialized organelles of intracellular catabolism (4). Indeed, an increase in hepatic lysosomes has previously been reported in rats during starvation (4) and during phlorizin-induced hypoglycemia (5), two situations in which hyperglucagonemia is reportedly present (6, 7).

The present study was designed to determine if the endogenous hyperglucagonemia and the hypoinsulinemia that occur in poorly controlled diabetes (8–10) are accompanied by an increase in number and volume of hepatic lysosomes and, if so, whether they can be reversed by insulin.

METHODS

Experimental procedures. Male Wistar rats weighing 160–209 g were employed in all experiments. Severe dia-

betes with ketoacidosis was induced by the rapid injection of 100 mg/kg of streptozotocin[1] into the tail vein of rats fasted for 18 h. This dose had been previously demonstrated by Junod, Lambert, Stauffacher, and Renold (11) to produce severe diabetic ketoacidosis, a state which is associated with striking hyperglucagonemia (12). For control purposes mild nonketotic diabetes was produced in another group of rats by injecting 50 or 60 mg/kg of streptozotocin. A nondiabetic control group was given only a saline placebo without streptozotocin. All rats had unlimited access to a standard Purina Formulab Chow diet (Ralston Purina Co., St. Louis, Mo.) and water. The rats consumed the food provided them until the time of sacrifice.

Animals were sacrificed by guillotine decapitation in a fully conscious state. Blood was collected in chilled tubes containing 500 U of Trasylol and 12 mg of ethylenediaminetetracetic acid per ml of whole blood. Specimens were immediately centrifuged and the plasma frozen at −20°C until the time of assay no more than 14 days later.

Analytical procedures. Plasma glucagon was measured by radioimmunoassay (13) as recently modified (14) and insulin by the method of Yalow and Berson (15), as modified by Herbert, Lau, Gottlieb, and Bleicher (16). Qualitative determinations of urinary glucose were made on each 24-h sample by using TesTape and acetone was tested with Acetest tablets.

Electron microscopy and morphometric methods. At the time of sacrifice, a portion of the right lobe of the liver was promptly excised, diced into small cubes, and fixed in a 4% glutaraldehyde solution in phosphate buffer (0.1 M, pH 7.4). The right lobe was chosen because of the ease of prompt excision, and the fact that no evidence of major differences from other lobes was observed. This method of fixation was selected because it gives results equal in quality to the more cumbersome perfusion method. After 2 h, the tissue was washed in the same buffer, postfixed in 2% osmium tetroxide for 2 h, dehydrated in graded series of ethanol, and embedded in Epon 812 (17). Sections 600–800 Å thick were cut with a diamond knife, and stained with uranylacetate and lead citrate (18).

Electron micrographs for morphometric study were recorded on 70-mm film in a Philips EM 300 electron microscope (Philips Electronic, Mount Vernon, N. Y.). A carbon grating replica with 2,160 lines per millimeter was recorded on each film for calibration. Films were examined in a table projector unit (19).

Sampling. Ultrathin sections from three randomly selected blocks per rat were examined and 36 micrographs (12 from each section) were randomly chosen as described by Stäubli, Hess, and Weibel (20). The magnification was 21,000 and a double lattice system with 9:1 ratio was used (21); the coarse lattice of heavy lines was used for evaluation of the cytoplasmic volume, lysosomes being estimated with the fine point grid.

Lysosomes were classified in three groups: *glycogenosomes,* defined as membrane-lined bodies containing glycogen; *dense bodies,* membrane-lined bodies containing electron-dense material without recognizable cytoplasmic elements; and *autophagosomes,* vacuoles containing a variety of cytoplasmic elements, such as mitochondria or endoplasmic reticulum in various stages of degeneration or breakdown. Only structures that clearly met the above definitions were counted, and questionable decisions were extremely rare. One of us (M. A.) carried out the measurements without knowledge of the experimental conditions. Typical examples of each type of structure counted are shown in Fig. 1.

Stereological methods. The volume density, V_V, of each type of lysosomes was determined by point counting according to the principles of Weibel (19, 21–23).

$V_V = P_P$ lysosomes/P_P cytoplasm. P_P represents the lattice points enclosed by a given profile.

RESULTS

Nondiabetic rats. Four nondiabetic rats were sacrificed 3 days after a placebo injection of streptozotocin-free saline. They had been without glycosuria or ketonuria and had gained an average of 3 g during this period. Fig. 2 indicates that plasma glucose, insulin, and glucagon were within the zone regarded as normal. The volume density of all lysosomes averaged 75×10^{-4} (SEM $\pm 7.0 \times 10^{-4}$). The mean volume density of glycogenosomes was 46.2×10^{-4} (SEM $\pm 4.0 \times 10^{-4}$), of dense bodies 17.1×10^{-4} (SEM $\pm 2.0 \times 10^{-4}$), and of autophagosomes 11.6×10^{-4} (SEM $\pm 1.7 \times 10^{-4}$) (Table I).

Severe diabetic ketoacidosis. In 6 rats with severe ketoacidosis induced by 100 mg/kg of streptozotocin there was severe glycosuria, polyuria, and ketonuria and an 18 g weight loss during the 3 days before sacrifice. (Fig. 3). At the time of sacrifice, plasma glucose averaged 462 mg/100 ml (SEM ±80), insulin 7.5 μU/ml (SEM ±0.9), and glucagon 403 pg/ml (SEM ±3.2), all significantly abnormal ($P < 0.001$). The molar ratio of insulin to glucagon (I/G) was 0.3 (SEM ±0.1), within an abnormally low zone observed previously only in so-called "catabolic" states. The volume density of all lysosomes averaged 222.8×10^{-4} (SEM $\pm 19.8 \times 10^{-4}$), significantly above the normal controls ($P < 0.0005$) (Fig. 3). Glycogenosomes were virtually absent in this group, with a volume density of only 2.7×10^{-4} (SEM $\pm 0.09 \times 10^{-4}$), significantly less than the control group ($P < 0.0005$). Volume density of dense bodies averaged 47.8×10^{-4} (SEM $\pm 5.0 \times 10^{-4}$) and autophagosomes 172.2×10^{-4} (SEM $\pm 20.0 \times 10^{-4}$), both significantly above the controls (Table I).

Mild diabetes. Seven rats were injected with 50 or 60 mg/kg of streptozotocin. As shown in Fig. 4, these rats exhibited only modest glycosuria and ketonuria, and did not lose weight. Plasma glucose averaged 281 mg/100 ml (SEM ±63), insulin 12.6 μU/ml (SEM ±2.3), glucagon 155 pg/ml (SEM ±16), and I/G 2.0 (SEM ±0.3), all within the normal range. Volume density of lysosomes averaged 77.6×10^{-4} (SEM $\pm 5.5 \times 10^{-4}$), not different from the normal group and significantly below the ketoacidotic group ($P < 0.0005$). The volume density of glycogenosomes, dense bodies, and autophagosomes averaged, respectively, 25.8×10^{-4} (SEM $\pm 3.1 \times 10^{-4}$), 29.0×10^{-4} (SEM $\pm 3.0 \times 10^{-4}$), and 22.8×10^{-4} (SEM $\pm 2.0 \times 10^{-4}$). Glycogenosomes

[1] Kindly supplied by Dr. William Dulin, The Upjohn Company, Kalamazoo, Mich.

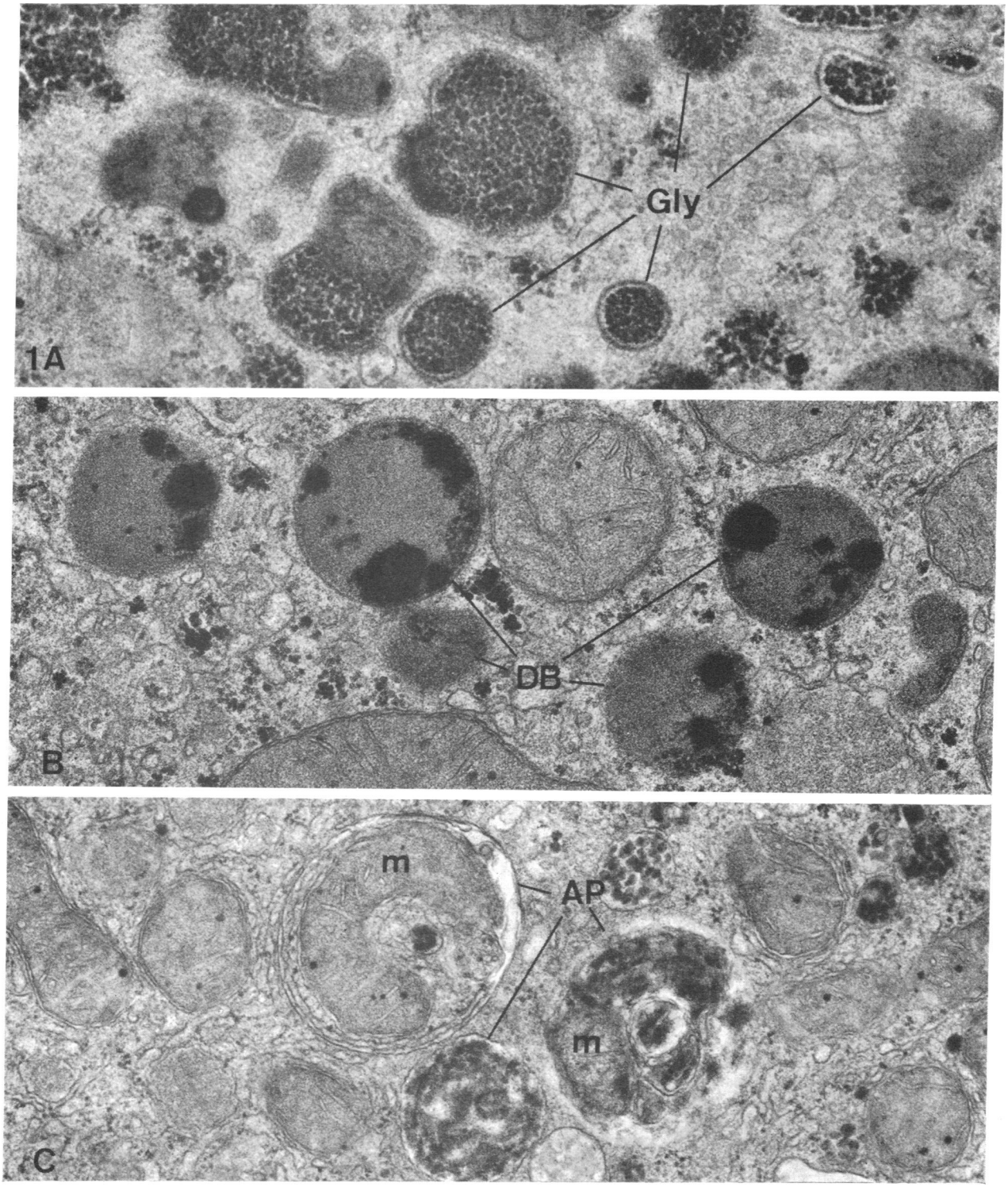

FIGURE 1 Typical examples of each class of lysosomes. (A) *Glycogenosomes* (*Gly*), membrane-lined bodies filled with glycogen (× 50,000). (B) *Dense bodies* (*DB*), membrane-lined bodies containing amorphous material of various electron density (× 42,000). (C) *Autophagosomes* (*AP*), vacuoles containing cell organelles (*m* = mitochondria) at various stages

were significantly below the controls ($P < 0.0005$), and dense bodies and autophagosomes were increased ($P < 0.0005$) (Table I).

Effect of insulin. To determine if correction of the hypoinsulinemia and hyperglucagonemia of severe diabetic ketoacidosis would reverse the hepatic autophagy, four such rats were given protamine zinc insulin subcutaneously as required for diabetic control. The average dose needed to prevent polyuria and heavy glucosuria was 4 U twice daily. As shown in Fig. 5, glycosuria and ketonuria were corrected by insulin, and the rats gained an average of 31 g during the period of insulin treatment. At the time of sacrifice, plasma glucose averaged 127 mg/100 ml (SEM ±4), insulin 156 μU/ml (SEM ±45), and glucagon 104 pg/ml (SEM ±14). The molar insulin: glucagon ratio was 40 (SEM ±12), far above the normal basal range. Volume density

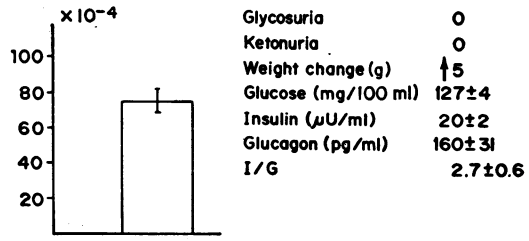

FIGURE 2 The volume density of hepatic lysosomes of four nondiabetic rats together with their laboratory data (mean ±SEM).

of lysosomes averaged only 37.4×10^{-4} (SEM $\pm 2.0 \times 10^{-4}$), significantly below the severely diabetic group, the mildly diabetic group, and even the normal controls ($P < 0.0025$). Thus, the administration of insulin was accompanied by a suppression of lysosomal volume density to below normal, a consequence of a reduction in

TABLE I

Volume Density × 10^{-4} ±SEM *of Hepatic Lysosomes in Normal, Untreated Diabetic, and Treated Diabetic Rats*

	Nondiabetic (n = 4)	Mild diabetes (n = 7)	Severe diabetic (n = 6)	Insulin-treated (n = 4)
All lysosomes	75.0±7.0	77.6±5.0	222.8±19.8	37.4±2.0
		NS	$P < 0.0005$	
			$P < 0.0005$	
		$P < 0.0005$		$P < 0.0005$
		$P < 0.0002$		
Glycogenosomes	46.2±4.0	25.8±3.0	2.7±0.09	5.2±0.07
			$P < 0.0005$	
	$P < 0.0005$	$P < 0.0005$		$P < 0.0005$
		$P < 0.0005$		
		$P < 0.0005$		
Dense bodies	17.1±2.0	29.0±3.0	47.8±5.0	22.2±2.0
			$P < 0.05$	
	$P < 0.0025$	$P < 0.0025$		
		$P < 0.0005$		$P < 0.0005$
		NS		
Autophagosomes	11.6±1.0	22.8±2.0	172.2±20.0	9.9±1.0
			$P < 0.0005$	
	$P < 0.0005$	$P < 0.0005$		$P < 0.0005$
	$P < 0.0005$			$P < 0.0005$
		NS		

of degradation and heterogeneous dense material considered to be end products of digestion (× 32,000).

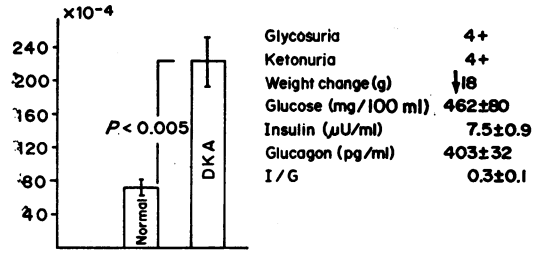

FIGURE 3 A comparison of volume density of hepatic lysosomes of six rats with severe diabetic ketoacidosis with that of four normal rats, together with the laboratory data of the former.

dense bodies and autophagosomes to normal with only a small, albeit significant, increase in glycogenososmes (Table I).

Relationship between lysosomes and hormones. To determine if a quantitative relationship between hormones and lysosomes could be demonstrated the lysosomal volume density was plotted as a function of the level of insulin, of glucagon, and of the molar insulin: glucagon ratio. There was a significant positive correlation between the volume density of lysosomes and plasma glucagon ($r = 0.79$; $P < 0.01$) but volume density was not significantly correlated with insulin ($r = 0.41$; $P > 0.1$) nor with the insulin: glucagon ratio ($r = -0.41$; $P > 0.1$) (Table I).

DISCUSSION

The results provide the first demonstration that in severe diabetes induced in rats by 100 mg/kg of streptozotocin there is a striking increase in the volume density of hepatic lysosomes when compared with nondiabetic and with mildly diabetic rats. Insulin treatment not only reverses this autophagy but reduces the lysosomal volume density to a level significantly below normal. Inasmuch as glycogenosomes were virtually absent in the severely diabetic group, the increase in lysosomes was exclusively a consequence of an increase in dense bodies and autophagosomes, primarily the latter. In mild diabetes without hyperglucagonemia, hypoinsulinemia, or other evidence of uncontrolled diabetes the lysosomal volume density was normal, although glycogenosomes were significantly reduced and dense bodies and autophagosomes significantly increased.

The mechanism by which the uncontrolled diabetic state induces an increase in these organelles is not revealed by these studies. However, there is circumstantial evidence implicating the hyperglucagonemia and hypoinsulinemia. First, in these studies there was a significant positive correlation between the total lysosomal volume density and the plasma glucagon level. Although there was no significant negative correlation between total lysosomal volume density and plasma insulin or the molar insulin: glucagon ratio, insulin dramatically reversed the hepatic autophagy. Second, it is well established that glucagon is capable of inducing hepatic autophagy (1–4). Third, other conditions in which glucagon is reportedly high in relation to insulin, namely starvation (6), and phloridzin diabetes (7), are also associated with increased hepatic autophagy (4, 5). However, the influence of other factors present in uncontrolled diabetes cannot be excluded by these studies, which do not necessarily establish a cause and effect relationship between hyperglucagonemia and hepatic autophagy. It is likely that insulin lack by itself would induce similar changes. It is possible that other abnormalities might also influence the development of hepatic autophagy.

A reduced concentration of insulin in the presence of a high glucagon level augments hepatic gluconeogenesis in the perfused rat liver preparation (24–28). This effect has been demonstrated even when amino acids are omitted from the perfusate (24), suggesting that hepatic amino acids are among the precursors. The increase in catabolic organelles in these studies could be regarded as a morphologic counterpart of a catabolic state in which the liver sacrifices its own proteins to produce fuel as the consequence of inappropriate signals from the islets of Langerhans.

A relatively low molar ratio of insulin to glucagon has also been reported in most patients with severe traumatic shock (29), severe burns (30), and severe infection (31), conditions in which a negative nitrogen balance is also known to be present. However, morphometric studies of lysosomes have not been conducted in these disorders. The clinical significance of hepatic

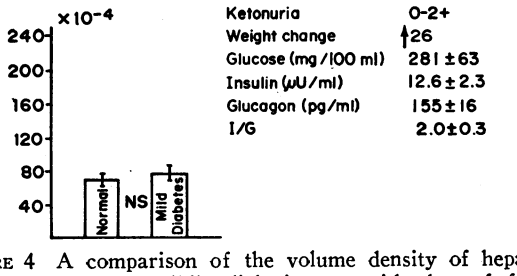

FIGURE 4 A comparison of the volume density of hepatic lysosomes of seven mildly diabetic rats with that of four nondiabetic rats, together the laboratory data of the former.

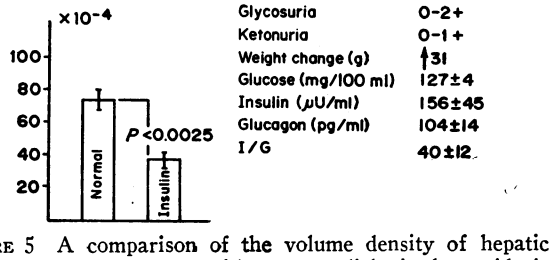

FIGURE 5 A comparison of the volume density of hepatic lysosomes in four rats with severe diabetic ketoacidosis after insulin treatment with that of four nondiabetic rats, together with the laboratory data of the former.

autophagy in diabetes and other catabolic states thus remains to be determined, but if it does indeed represent a morphologic expression of augmented hepatocellular autodigestion, treatment with insulin and glucose might provide a rational means of anticatabolic therapy.

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