Effect of streptozotocin-diabetes on rat liver mitochondrial adenosine triphosphatase turnover

Antonio JORDÁ,* Ernesto PÉREZ-PASTOR and Manuel PORTOLÉS

Instituto de Investigaciones Citológicas de la Caja de Ahorros de Valencia, Amadeo de Saboya 4, 46010 Valencia, Spain

The apparent turnover rates of some mitochondrial enzymes can be modified in diabetes. We studied the effect of streptozotocin-diabetes on the half-life of a protein tightly bound to the inner membrane, ATPase. The half-life ($t_{1/2}$), measured by the double-isotope technique, decreased by approx. 20% in diabetes (from ≈ 2.56 days in controls to ≈ 2.06 days in diabetic rats). These results suggest that diabetes produces an increase in degradation of ATPase by a mechanism which is not yet clear, possibly influenced by alterations induced by diabetes in hepatic lysosomes that are associated with hepatic autophagy.

INTRODUCTION

Dice *et al.* (1978) have reported that there are some fundamental changes in the degradation of cellular proteins in streptozotocin-induced diabetes; the average rates of protein catabolism are accelerated by 30–150% in liver of insulin-deficient rats. Furthermore, there is a general correlation of M_r and pI of proteins with the half-life; that is, large proteins should be degraded more rapidly than small proteins, and acidic proteins appear to be degraded more rapidly than neutral or basic proteins (Goldberg & St. John, 1976). However, Bond (1980) found, in a milder form of insulin-dependent diabetes, a general decrease in the incorporation of radiolabelled amino acids in the diabetic state, with little if any change in the liver proteolytic machinery. The fundamental changes in protein degradation seen in severe diabetes were not observed in a less severe form of the disease.

Mitochondrial ATPase (ATP phosphohydrolase, EC 3.6.1.3) is an enzymic protein that plays both a structural and a catalytic role in oxidative phosphorylation and other energy-linked reactions in mitochondria; the ADP–ATP exchange is in fact catalysed by ATPase in intact membrane systems (Catterall & Pedersen, 1971). The half-life of mitochondrial ATPase, which has a high M_r and a low pI (Catterall & Pedersen, 1971), could theoretically be affected in an insulin-deficient state (Dice *et al.*, 1978; Goldberg & St. John, 1976).

Because of the important role of mitochondrial ATPase activity in the energy metabolism of the cell, and because of the effect that severe diabetes can exert on the general turnover of proteins, we have studied the turnover of this enzyme in streptozotocin-induced diabetic rats. We have found decreases in the half-life of liver mitochondrial ATPase in the severe diabetic state. The possible physiological significance of these findings is discussed.

MATERIALS AND METHODS

Rats

Male Wistar rats (180–230 g) were used; they were maintained on a standard diet [20% (w/w) protein] *ad libitum*, and had free access to water.

Chemicals

Standard analytical-grade reagents were obtained from Sigma (St. Louis, MO, U.S.A.) and Boehringer (Mannheim, Germany). L-[U- 14 C]Leucine (324 Ci/mol) and L-[4,5- 3 H]leucine (60 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.); the latter was diluted before injection with unlabelled leucine in order to inject equal quantities of leucine.

Diabetes

Severe insulin-dependent diabetes was induced in rats by intraperitoneal injection of a single dose of streptozotocin (Jordá *et al.*, 1982). The rats were kept in wire metabolic cages and their diabetes was monitored by measurements of body weight, food and water intake, urine volume, urine and blood glucose (Jordá *et al.*, 1981) and serum insulin (solid-phase 125 I radioimmunoassay, with insulin standard from Novo, Copenhagen, Denmark).

Administration of labelled compounds

At 8 days after induction of experimental diabetes (Jordá *et al.*, 1982), rats were fasted for 16 h, and then each rat was injected intraperitoneally with 25 μ Ci of [14 C]leucine in 1 ml of 0.9% NaCl, as recommended by Glass & Doyle (1972) (food was restored 4 h later). Some rats were injected at the same time with 75 μ Ci of [3 H]leucine in 1 ml of 0.9% NaCl, whereas the others received this at 3 days or 10 days after the first injection (the previous fasting regimen mentioned above was repeated). The rats were killed 4 h after the injection of [3 H]leucine. Radioactive proteins in cell fractions were precipitated with 5% (v/v) trichloroacetic acid and washed by the procedure of Nicoletti *et al.* (1977). The proteins were dissolved in 0.2 M-NaOH, neutralized with HCl, and samples were placed in scintillation vials. Sample volumes were adjusted to 1 ml with water, and 10 ml of scintillation liquid [1 litre of toluene, 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 5 g of 2,5-di-phenyloxazole, 0.5 litre of Triton X-100] was added.

For counting radioactivity of the purified enzyme, the enzyme protein was subjected to polyacrylamide-gel electrophoresis. Enzyme was identified on the gels by

* To whom reprint requests should be addressed.

protein and enzyme-specific staining (Chayen *et al.*, 1973); the portions containing the enzyme were cut out, combined in vials and dissolved by addition of 200 μ l of H_2O_2 and heated for 16–24 h at 50 °C. To each 1 ml of distilled water and 10 ml of scintillation fluid were added.

Radioactivity was measured in an Intertechnique model SL 4000 automatic scintillation counter. Efficiencies were approx. 30% for 3H and 50% for ^{14}C .

Liver protein synthesis

Cell-free protein synthesis with rat liver extracts was carried out as described previously (Felipo & Grisolia, 1985; Felipo, 1986), with [3H]leucine (1 Ci/mmol).

Cell fractionation and isolation of specific proteins

All steps were carried out at 0–4 °C unless otherwise stated. Rats were killed by decapitation. The livers were excised, rinsed with 0.25 M-sucrose, minced and homogenized in 10 vol. (v/w) of 0.25 M-sucrose in a Potter–Elvehjem homogenizer fitted with a Teflon pestle. The mitochondrial, cytosol and microsomal fractions were isolated as follows. The homogenate was centrifuged at 600 g for 5 min in a Sorvall Superspeed RC-5 centrifuge. The sediment was discarded and the supernatant was centrifuged again at 14000 g for 10 min. The sediment containing the mitochondria was washed with 2 $\times$ 2 ml of 0.25 M-sucrose/g of tissue. Cytosol and microsomal fractions were obtained by centrifuging the mitochondrial supernatant at 105000 g in a Beckman L5-65 centrifuge for 60 min. The lipid layer of the cytosol was discarded. Samples of all fractions were kept for determination of radioactivity.

The protein content of the mitochondrial sediment was determined by the biuret/deoxycholate method (Jacobs *et al.*, 1956), with bovine serum albumin as standard.

Purification of ATPase

Enzyme was purified as described by Catterall & Pedersen (1971), with minor modifications. The fractions containing the bulk of the enzyme activity from the DEAE-cellulose column were pooled and the protein was precipitated with $(NH_4)_2SO_4$ (final concn. 65% satn.) overnight at 4 °C. After centrifugation (15 min, 27000 g) the pellet was dissolved in a small volume of 50 mM-Tris/ H_2SO_4 (pH 7.8)/2 mM-ATP/1 mM-EDTA. Any insoluble precipitate was removed by centrifugation

(15 min, 27000 g). To the supernatant ATP was added to a final concn. of 12 mM, and saturated $(NH_4)_2SO_4$, pH 7.2, to give 50% saturation. With this method, we were able to store the enzyme at 4 °C for more than 2 months without appreciable loss of activity.

Other determinations

Leucine was measured by .h.p.l.c. as described by Portolés *et al.* (1985) by using a reverse-phase Resolve C-18 column (5 μ m) from Waters–Millipore, glucose as described by Werner *et al.* (1970), and protein as described by Lowry *et al.* (1951), except in crude fractions, when the method of Jacobs *et al.* (1956) was used.

Statistical

All results have been submitted to statistical analysis, and the degree of significance was calculated by Student's *t* test.

RESULTS AND DISCUSSION

Table 1 shows the main parameters measured in control and diabetic rats at time of killing, 11 days after induction of streptozotocin-diabetes. Both serum glucose and insulin indicate the severity of diabetes in the experimental rats; diabetic rats also lost approx. 20% of their body weight, although their food intake increased by approx. 30%, which contrasts with the studies by Bond (1980), in which mice maintained body weight by increasing their food intake, and those by Dice *et al.* (1978), in which diabetic rats lost approx. 30% of their body weight in 3 days, whereas starved rats lost approx. 25% in the same period. The mitochondrial protein of liver of diabetic rats increased, although no changes were observed in protein concentration per g of liver, or per whole liver; this fact has previously been considered to be due to a general increase in urea-cycle enzymes in diabetes (Jordá *et al.*, 1981, 1982) and particularly of carbamoyl-phosphate synthase I, which represents 15–20% of the mitochondrial protein (Cohen, 1970; Clarke, 1976; Lusty, 1978; Zaragoza *et al.*, 1987). The same parameters were measured 18 days after induction of experimental diabetes, and no differences were observed except a slight decrease in body weight (results not shown).

Purification of ATPase yielded an enzyme which gave a well-defined band in gels stained for protein with Coomassie Blue. The double-isotope method is best applied to proteins with a $t_{1/2}$ (half-life) in the range 2–8

Table 1. Effects of streptozotocin-diabetes on some general parameters

The results are expressed as means $\pm$ S.D. for six to eight rats: **P* < 0.001; ***P* < 0.01.

	Control	Diabetic	Difference (%)
Glucose (mg/dl)	157 $\pm$ 21	632 $\pm$ 33*	400
Insulin (μ -i.u./ml)	56 $\pm$ 7	6 $\pm$ 2*	–90
Body wt. (g)	220 $\pm$ 20	175 $\pm$ 31**	–20
Liver wt. (g)	6.8 $\pm$ 0.6	6.0 $\pm$ 0.5	–12
Protein/g of liver (mg)	243 $\pm$ 23	250 $\pm$ 22	
Protein/whole liver (mg)	1652 $\pm$ 54	1500 $\pm$ 62	
Mitochondrial protein (mg/g of liver)	53 $\pm$ 8	65 $\pm$ 7**	22
Food intake (g/day)	19 $\pm$ 5	25 $\pm$ 6**	30
Fluid intake (ml/day)	22 $\pm$ 4	54 $\pm$ 7*	245

Table 2. $^3\text{H}/^{14}\text{C}$ ratios, rate constants of degradation and apparent half-lives of rat liver cell fractions and ATPaseResults are expressed as means $\pm$ S.D. for six rats.

Fraction	Interval between injections					
	3 days			10 days		
	$^3\text{H}/^{14}\text{C}$	K_d (days $^{-1}$)	$t_{1/2}$ (days)	$^3\text{H}/^{14}\text{C}$	K_d (days $^{-1}$)	$t_{1/2}$ (days)
Homogenate	5.3 ± 0.3	0.19	3.5	9.9 ± 0.3	0.20	3.4
Mitochondria	4.6 ± 0.2	0.13	5.0	5.2 ± 0.4	0.10	6.7
Cytosol	5.2 ± 0.4	0.18	3.9	10.0 ± 0.3	0.20	3.4
Microsomal	6.7 ± 0.4	0.27	2.4	14.0 ± 0.3	0.26	2.5
ATPase	6.6 ± 0.3	0.27	2.6	15.2 ± 0.4	0.27	2.6

days (for mitochondrial ATPase the approximate half-life was determined in a preliminary experiment; Pérez-Pastor *et al.*, 1982). We chose 3- and 10-day periods for our experiments, and, as illustrated in Table 2, our data for liver homogenate and cell fractions from control rats agree with those obtained by Glass & Doyle (1972). A direct influence of the amount of dietary protein and diabetes on the amounts of urea-cycle enzymes of rat liver has been demonstrated previously (Nicoletti *et al.*, 1977; Jordá *et al.*, 1981, 1982; Zaragoza *et al.*, 1987). In Table 3 we show $^3\text{H}/^{14}\text{C}$ ratios, K_d and $t_{1/2}$ values for cell fractions and ATPase from diabetic rats, at both 3- and 10-day intervals, compared with controls (Table 2). K_d values for cellular fractions of diabetic rats were increased slightly at 3 days, and more significantly in the 10-day experiments. The $t_{1/2}$ calculated for the isolated enzyme from diabetic rats was some 20% less than for control rats (approx. 2.06 days and 2.57 days respectively). No changes were observed in the specific radioactivity of the hepatic leucine pool during the diabetic state compared with the control state, since no changes in liver content of leucine were observed between control rats and experimental rats at several intervals after induction of diabetes (Table 4).

As described in the Materials and methods section, rats were injected with [^{14}C]leucine at 8, 11 and 18 days after induction of diabetes, and with [^3H]leucine at 8 days

(simultaneously with the [^{14}C]leucine), and at 11 and 18 days (days 3 and 10 after the first injection of [^{14}C]leucine) for the studies of the half-life of ATPase. Coincidentally, cell-free extracts of livers of diabetic rats taken at 8, 11 and 18 days after the induction of diabetes were used for studies of protein synthesis, using [^3H]leucine (1 Ci/mmol). The rate of protein synthesis did not change in the diabetic state during the interval studied, as shown by the results in Table 4, nor were there variations in the incorporation of leucine into proteins in this interval.

As postulated by Dice *et al.* (1978), there is a correlation between protein size and pI and the half-life of liver protein in starvation and in diabetes. The effects of starvation, however, appear to be less drastic than those of diabetes, suggesting that there may be a more extreme imbalance of hormones and nutrients in diabetes. ATPase is a protein with a high M_r and acidic character (Catterall & Pedersen, 1971) and, as shown in Table 3, its half-life was decreased in the diabetic state. This could be in agreement with the postulate by Dice *et al.* (1978), although data reported by Nicoletti *et al.* (1977) showed that it did not apply for all rat liver enzymes. On the other hand, Bond (1980), who did not observe these changes in a less severe form of diabetes, suggested that in their experimental mice the effect of insulin deficiency on protein catabolism may have been counterbalanced

Table 3. Effect of streptozotocin-diabetes on the $^3\text{H}/^{14}\text{C}$ ratios, rate constants of degradation and apparent half-lives of rat liver cell fractions and of ATPaseResults are expressed as means $\pm$ S.D. for six rats.

Fraction	Interval between injections					
	3 days			10 days		
	$^3\text{H}/^{14}\text{C}$	K_d (days $^{-1}$)	$t_{1/2}$ (days)	$^3\text{H}/^{14}\text{C}$	K_d (days $^{-1}$)	$t_{1/2}$ (days)
Homogenate	6.3 ± 0.3	0.21	3.1	15.9 ± 0.4	0.31	2.6
Mitochondria	5.5 ± 0.2	0.13	5.6	10.6 ± 0.5	0.19	3.4
Cytosol	5.5 ± 0.4	0.20	3.5	19.9 ± 0.6	0.35	2.3
Microsomal	6.6 ± 0.4	0.22	3.1	20.0 ± 0.5	0.38	2.1
ATPase	7.7 ± 0.3	0.33	2.06	11.8 ± 0.4	0.22	2.07

Table 4. Effect of streptozotocin-diabetes on liver protein synthesis and amount of leucine in liver

Protein synthesis is calculated from the radioactivity incorporated into protein, the specific radioactivity of [^3H]leucine used (1 Ci/mmol, 1 mCi/ml), the concentration of [^3H]leucine in the synthesis mixture (133 μM) and the concentration of unlabelled leucine present in the extract (Felipo & Grisolia, 1985; Felipo, 1986). To measure the content of leucine in liver, a portion of liver was quickly removed, frozen in liquid nitrogen, and leucine was determined by h.p.l.c. (Portolés *et al.*, 1985). Results are expressed as means $\pm$ s.d. for four to six rats.

	Time after induction of diabetes (days)			
	0	8	11	18
Leucine incorporated into protein (fmol/mg of protein)	—	364 $\pm$ 72	354 $\pm$ 65	368 $\pm$ 83
Unlabelled leucine in liver extracts (nmol/g of liver)	242 $\pm$ 74	251 $\pm$ 82	246 $\pm$ 67	239 $\pm$ 65
Leucine in liver (nmol/g of liver)	157 $\pm$ 35	162 $\pm$ 40	155 $\pm$ 42	165 $\pm$ 38

by the increase in amino acids available to the liver, owing to increased food intake, and further suggested that perhaps the abnormal characteristics of protein degradation observed in diabetic rats by Dice *et al.* (1978) may have been due to starvation rather than to the diabetes itself. This obviously was not the case in our diabetic rats, since, despite the increase in food intake, and contrary to the findings of Bond (1980), there was an increase in protein degradation. There are probably other more complex factors which can also intervene in these processes.

Turnover of mitochondrial proteins appears to be a process involving lysosomal and intramitochondrial neutral proteinases (Grisolia *et al.*, 1980); it has also been demonstrated that ATPase is inactivated by components of the mitochondrial inner membrane in the presence of lysosomes (Soler *et al.*, 1980; Timoneda *et al.*, 1981). Moreover, there is good evidence that there are alterations in hepatic lysosomes associated with hepatic autophagy in some forms or stages of diabetes (Amherdt *et al.*, 1974); it is possible that an increase in intracellular protein breakdown accompanies these more severe forms of diabetes where lysosomal changes are observed.

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