Inhibition of cellular autophagy in proximal tubular cells of the kidney in streptozotocin-diabetic and uninephrectomized rats

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Summary. To examine the significance of anti-catabolism in renal hypertrophy, cellular autophagy was investigated by electron microscopic morphometry in proximal tubular cells (PTCs) of the outer cortex of the rat kidney after the induction of diabetes mellitus by streptozotocin (STZ) and after unilateral nephrectomy. Adult male Sprague-Dawley rats were divided into three groups and killed by retrograde perfusion fixation, 1, 2 and 3 days after the induction of diabetes (group D; n=24), after unilateral nephrectomy (group N; n=24) and after combined treatment (group DN; n=24). Untreated, age-matched litter mates served as controls (group C; n=24). By comparison with these controls, the left kidney to initial body weight ratio was increased by 8, 23, and 15% in group D animals, by 8, 23, and 24% in group N animals, and by 10, 21, and 25% in group DN animals at the first, second and third day, respectively. Quantitative evaluation of large test areas showed that the volume and numerical densities of autophagic vacuoles (AVs) in PTCs were significantly lower in these hypertrophied kidneys than in the controls. The average reduction in AV volume density was about 65% in group D animals, about 50% in group N animals and about 75% in group DN animals. These data show that autophagic degradation of cytoplasmic components in PTCs is inhibited in renal hypertrophy independently of the growth stimulus, i.e. uninephrectomy or diabetes. Since insulin per se inhibits cellular autophagy in PTCs, the expected effect of insulin deficiency seems to be counteracted by as yet undefined stimuli that may be related to metabolic work load.

Key words: Cellular autophagy – Ultrastructural morphometry-Proximal tubules – Adaptive hypertrophy – Uninephrectomy-Experimental diabetes

Introduction

Cellular autophagy is a well known process whereby cytoplasmic constituents are enclosed by membranes and sequestered into the lysosomal compartment for eventual digestion (Pfeifer 1976; Marzella and Glaumann 1980; Glaumann et al. 1981; Glaumann and Ahlberg 1987). Autophagic vacuoles (AVs) are formed under physiological conditions in PTCs of the kidney and in liver cells (Pfeifer and Warmuth-Metz 1983). The importance of AVs for intracellular protein degradation has been convincingly shown in numerous experiments (Seglen et al. 1979; Furano et al. 1982; Henell and Glaumann 1984; Henell et al. 1987). In addition, quantitative electron microscopic studies (Pfeifer and Bertling 1977; Pfeifer and Jurilj 1979; Dämmrich and Pfeifer 1983; Jurilj and Pfeifer 1985, 1990; Müller et al. 1987) have demonstrated that inhibition of the autophagic process, expressed morphologically by a reduction in the volume and numerical densities of AVs, plays a prominent role in induced growth processes (for review, see Pfeifer 1982).

Renal enlargement, a model of localized tissue growth, has been interpreted as a response by the kidney to an increased work load after uninephrectomy (Hayslett 1979; Hostetter et al. 1981; Chevalier 1982; Finn 1982; Fleck and Bräunlich 1984; Fine 1986), and similar considerations evolved from the observations of kidney growth in early experimental and human diabetes (Mogenssen et al. 1979, 1981; Hirose et al. 1980; Jensen et al. 1981; Seyer-Hansen 1983; Osterby 1986; Viberti and Wiseman 1986; Bank et al. 1988; Flyvbjerg et al. 1988; Kleinman and Fine 1988; Kunjara et al. 1988). Additive effects on renal enlargement in rats have been found when experimental diabetes and uninephrectomy were combined (Ross and Goldman 1971; Seyer-Hansen 1978), but at present the mechanisms responsible for kidney growth in diabetic rats or compensatory growth after uninephrectomy are only partially understood (Rasch and Rytter-Nørsgaard 1983).

Since previous studies have shown that insulin inhibits the formation of AVs in renal PTCs (Pfeifer and Warmuth-Metz 1983) the question arises whether insulin deficiency in STZ diabetes leads to the expected enhancement of cellular autophagy in the kidney, or whether cellular autophagy is inhibited as in other experimental models of organ hypertrophy (Hill and Malamud 1974; Dämmrich and Pfeifer 1983; Müller et al. 1987; Jurilj and Pfeifer 1990).

Materials and methods

Animals. A total of 96 male Sprague-Dawley SPF rats (D. Wenzel, Detmold, FRG), weighing 200–250 g at the start of the experiment, were used. They were acclimatized for at least 7 days to the housing conditions with constant temperature (22° C) and automatic light control (dark between 7:00 p.m. and 7:00 a.m.). The rats were housed two in each cage and were fed standard commercial rat pellets (ALTROMIN 1324, Altromin GmbH, Lage, FRG). Food and water were given ad libitum.

Experimental conditions. Because of the well-known circadian variations in cellular autophagy (Pfeifer and Scheller 1975), the experiments were restricted to intervals between 10:00 a.m. and 12 noon, requiring repeated investigation each involving a limited number of animals. In these single experiments at least one animal was included from each group, i.e. diabetic (D), unilateral nephrectomised (N), combined diabetic and unilateral nephrectomised (DN), and untreated age-matched controls (C). The final number of animals was 24 in each group.

Diabetes was induced by a single intravenous injection of STZ (Serva, Heidelberg, FRG), 65 mg/kg body weight, into the tail vein. Rats with blood glucose levels above 400 mg/dl were considered diabetic. Rats that failed to show an elevated blood glucose level were excluded from the study.

Right-sided nephrectomy, leaving the adrenal gland undamaged, was performed through a flank incision under sodium pentobarbital anesthesia (Nembutal, Ceva, Bad Segeberg, FRG), 40 mg/kg body weight given intraperitoneally. In the DN group, nephrectomy was performed immediately after the STZ injection.

Vascular perfusion fixation method. The animals were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The abdominal cavity was opened by a midline incision with lateral extensions. The aorta just below the celiac artery was carefully exposed and a loose ligature placed around it. The superior mesenteric artery was clamped and the aorta below the origin of the renal arteries was exposed and freed from adipose tissue. An injection valve three-way stopcock (Mecath Inc, 17G/1.4 mm O.D. × 40 mm, Codan Steritex Aps, Højvangen, Espergaerde, Denmark) was inserted towards the heart into the lumen of the aorta about 1 cm from its bifurcation. Blood samples were taken and collected in Monovette KF tubes (Sarstedt, Mümbrecht, FRG) and stored at 4° C until analyzed (approximately 2 h later). The ligature around the aorta was then rapidly tightened and the inferior vena cava was cut. Immediately after the perfusion had started the aorta was clamped just above the celiac artery.

By use of the three-way stopcock the perfusion system permitted switching from the rinsing to the fixative solution without a fall in perfusion pressure, which was kept at 148 cm H₂O (about 40 mmHg). The kidneys that failed to blanch immediately after the start of the perfusion were discarded. Each animal was first perfused with about 60 ml of the rinsing solution for 60 sec and then with 15 ml of the fixative for 3 min. The rinsing solution consisted of 0.08 M sodium cacodylate buffer containing 4% dextran 35 (Serva, Heidelberg, FRG) and 3.5% procaine (Serva, Heidelberg, FRG), pH 7.3, 370 mOsm. The fixative consisted of 3%

glutaraldehyde in an 0.08 M sodium cacodylate buffer containing 4% dextran 35, pH 7.3, 460 mOsm. The time required between laparotomy and the start of perfusion was less than 4 min.

Tissue preparation. After perfusion fixation the left kidney was excised and weighted. Cuboidal specimens (side length 1–1.5 mm) were cut from the outer cortex. These specimens were immersed for 2.5 h in the same fixative used for perfusion, then rinsed in 0.08 M sodium cacodylate buffer and postfixed in 2% osmium tetroxide in 0.14 M phosphate buffer (Millonig 1962). They were then rinsed, dehydrated and embedded in Epon 812 (Luft 1961).

Sections were cut with a diamond knife on a LKB Ultratome UM3. Semithin sections were cut from three blocks at 1 µm and stained with methylene blue-azur II (Richardson et al. 1960). One block from each animal was chosen and an area containing convoluted tubules was selected and trimmed for electron microscopy. Ultrathin sections (50–70 nm) were stained at room temperature with uranyl acetate and with a mixture of lead citrate, acetate and nitrate. The specimens were examined and photographed in a Siemens Elmiskop 101 electron microscope, operated at an acceleration voltage of 80 kV.

Morphometry. Autophagic vacuoles were identified according to the criteria of Pfeifer (1978) and counted directly on the EM screen. The square openings of the supporting copper grid (SCI G 200 HS cu 3.05 mm, Science Services, Munich, FRG), which were completely covered by the section, were evaluated for AVs. Six fields per animal were viewed systematically for AVs after scanning at a primary magnification of 20,000 ×.

By taking an electron micrograph at a primary magnification of 840X, the total profile surface area of the proximal convoluted tubules in each square opening evaluated could be calculated. The fractional area covered by the PTCs was determined at a final magnification of 2100 ×. An average profile surface area of PTCs per animal of 8700 ± 1200 µm² of PTCs was evaluated. The total area of cytoplasm examined for AVs in this study was about 8.4 × 10⁵ µm².

Electron micrographs of all the AVs counted were taken at a primary magnification of 20,000 × and were evaluated at a final magnification of 50,000 ×.

The area of the sectioned proximal tubules and the area of the AV profiles photographed during the evaluation were measured by digitized morphometry using an IBM PC-AT computer and the Sigma-Scan Measurement System (Jandel Scientific, San Francisco, USA). The photomicrographs were placed on a digitizing opaque tablet and the structural contours traced with a cursor connected to the microprocessor to compute the respective areas in µm². Latex particles of 1.05 µm in diameter (Unisphere Latex, Serva, Heidelberg, FRG) and the bars of the copper grids supporting the specimens were used for the calibrations. The diameter of the bars from 15 randomly sampled grids were measured with an ocular micrometer by light microscopy. The mean thus obtained was 10.036 (±0.547) µm.

The AV volume density [V_v (AV/PTC)] was calculated as the quotient of the total AV profile area to the total area of the sectioned PTCs per animal. The number of AVs per unit volume of PTCs [N_v (AV/PTC)] was assessed by applying the formula of Weibel (1969). A spherical shape and a normal size distribution were assumed for this calculation.

Analytical procedure. Blood glucose was analyzed in the Institut für Klinische Biochemie, University of Bonn (Head: Prof. Dr. F. Bidlingmeier), by a hexokinase-glucose-6-phosphate dehydrogenase method (Merck System Glucose, Merck, Darmstadt, FRG) adapted for the EPOS Analyser 5060 (Eppendorf, Hamburg, FRG).

Statistical methods. An analysis of variance (ANOVA) and the U-test were performed with SAS software (SAS Institute, Heidelberg, FRG) and used to test the statistical significance. The limit of significance was set at $p < 0.05$.

Results

The results of the blood glucose analysis are plotted in Fig. 1. The blood glucose levels were significantly increased in the diabetic rats (groups D and DN), the mean value being $466 (\pm 47)$ mg/dl, compared with the value found in the non-diabetic rats (groups N and C) of $160 (\pm 20)$ mg/dl ($p < 0.0001$). Less than 5% of the animals that received STZ failed to show an elevated blood glucose level. The elevated glucose levels in the STZ-diabetic rats were fairly well maintained during the 3-day experimental period.

The average body weight did not change significantly within the first 3 days of the experiment. While a slight loss of body weight was noted in groups D and DN, none could be detected in group N.

The kidney weights determined after the fixation procedure were found to be increased in the D, N, and DN groups. When these changes were expressed as the ratio of the left kidney to the initial body weight, there was a substantial increase of 8% on the first, 23% on

the second and 15% on the third day for group D animals, a gain of 8%, 23% and 24% for the group N animals, and an increase of 10%, 21% and 25% for the group DN animals, respectively, as compared with the corresponding controls. However, the ratios between kidney weight and body weight were not significantly different between the STZ-diabetic rats (groups D and DN) and the nephrectomized rats (group N) during the entire experimental period (Fig. 2).

Qualitative morphological findings

A uniform and reproducible preservation of the proximal tubules with open lumina, evenly arranged brush border zones, and little or no apical swelling was obtained by the method applied. The occurrence of a brush border, of endocytic vesicles and vacuoles, of large lysosomal bodies, and of microbodies was taken to indicate a proximal tubular cell. No attempt was made to distinguish between the different segments of the proximal tubule.

Fig. 1. Effects of STZ injection and/or unilateral nephrectomy on blood glucose levels (mg/dl). Values are means $\pm$ standard deviation ($n=8$). Asterisks denote statistically significant differences (U -test) ■ Control; ▨ nephrectomy; □ diabetes; ▩ diabetes and nephrectomy

Fig. 2. Effects of STZ-injection and/or nephrectomy on ratios of left kidney weight to initial body weight. Values are means $\pm$ standard deviation ($n=8$). Asterisks denote statistically significant differences (U -test) between the experimental groups and the corresponding control groups. For symbols see legend of Fig. 1

Fig. 3a-d. Different types of AVs in early stages. These are classified according to their contents: **a** A mitochondria with well preserved cristae. Apparently the AV and two probable lysosomes are fusing. **b** A portion of cytoplasmic ground substance together with a peroxisome (=microbody) identified by its single bordering membrane, its homogeneous matrix, as well as by cylindric struc-

tures (arrows) which have been sectioned transversely. **c** A mitochondria and cytoplasmic ground substance together with small cisternae and vesicles. **d** AV containing a portion of cytoplasmic ground substance surrounded by the segregating structure which appears as a multiple membrane. **a-d** $\times 50,000$

The AVs were defined as areas of still recognizable cytoplasm, limited by a single or double membrane from the remaining cytoplasm, and were found in all regions of the tubular cells. When the segregated cytoplasm could no longer be clearly identified because of lytic changes, the profiles were excluded from the evaluation.

The AVs encountered during the systematic evaluation did not show qualitative morphological changes related to either the experimental condition or the time interval. The segregated cytoplasmic components in the proximal tubular cells of the rat kidney consist of mitochondria (AVm), peroxisomes (AVp), and, taken as one type, of ground substance, glycogen particles, profiles

of endoplasmic reticulum and of the Golgi-apparatus (AVergs). Some AVs with "mixed" contents, or surrounded by multiple limiting membranes, were also noted (Fig. 3a-d).

Quantitative results. The volume density in the proximal tubular cell cytoplasm of all the types of AVs is shown in Fig. 4 and in Table 1. When compared with controls, the volume density was found to be significantly decreased after STZ-injection (groups D and DN) and after nephrectomy (group N). The volume density of the AVs was lower in the animals after STZ-injection (group D) than after nephrectomy (group N), but this

Fig. 4. Volume densities of total AVs [V_v (AV/PTC)] in proximal tubular cells. The mean values $\pm$ standard deviation (indicated by the vertical bars) of eight animals are shown. Asterisks denote statistically significant differences (*U*-test). For symbols see legend of Fig. 1

Fig. 5. Numerical densities of total AVs [N_v (AV/PTC)] in proximal tubular cells. The mean standard deviation (indicated by the vertical bars) of eight animals are shown. Asterisks denote statistically significant differences (*U*-test). For symbols see legend of Fig. 1

Table 1. AV parameters in PTCs. Mean values and standard deviation of the volume density (V_v), and the numerical density (N_v) of total AVs are listed for controls and experimental groups. Calculation of percentage change: (test group minus controls) $\times$ 100/controls

Parameter	DAT	Group		%C	N	%C	DN		VA
		C	D				DN	%C	
V_v (AV/PTC) ($\times 10^{-4}$)	1	1.07 $\pm$ 0.12	0.37 $\pm$ 0.16	-65	0.54 $\pm$ 0.16	-50	0.24 $\pm$ 0.11	-78	*
	2	1.09 $\pm$ 0.13	0.38 $\pm$ 0.13	-65	0.52 $\pm$ 0.20	-52	0.24 $\pm$ 0.09	-78	*
	3	1.00 $\pm$ 0.05	0.35 $\pm$ 0.07	-65	0.56 $\pm$ 0.12	-44	0.27 $\pm$ 0.13	-73	*
N_v (AV/PTC) ($\times 10^4$ /mm ³)	1	248 $\pm$ 72	80 $\pm$ 51	-68	83 $\pm$ 45	-66	50 $\pm$ 35	-80	*
	2	214 $\pm$ 36	52 $\pm$ 36	-76	87 $\pm$ 47	-59	51 $\pm$ 42	-76	*
	3	289 $\pm$ 91	84 $\pm$ 49	-71	72 $\pm$ 31	-75	53 $\pm$ 16	-82	*

DAT, Days after treatment; C, controls; D, diabetes; N, nephrectomy; DN, diabetes+nephrectomy; VA, variance analysis; %-C, percent of change; PTC, proximal tubular cell; * $P < 0.0001$

difference was statistically significant only at the third day. In contrast to this observation, the difference in the volume density of AVs between the DN and the N groups was already statistically significant after the

first day. However, no difference could be found between groups D and DN.

The total AV-compartment in the control group was composed of 56 ($\pm$ 2)% ergs, 38 ($\pm$ 1)% mitochondria, and

6 (± 2)% peroxisomes. This was in agreement with the data described in detail in rat PTCs in an earlier paper (Pfeifer and Scheller 1975). No substantial differences were noted in the composition of the AV compartment between the control and experimental groups.

As seen in Fig. 5 and Table 1, the numerical, like the volume density of the AVs is significantly reduced in the experimental groups (D, N and DN) compared with controls. There were, however, no major differences between the STZ-diabetic and nephrectomized rats during the 3-day experimental period.

Discussion

Compared with other organs and tissues, the kidney appears to be exceptional in that it shows marked growth in the early stages of diabetes mellitus, thus contrasting with the general pattern of protein loss which characterizes this metabolic disorder (Pain and Garlick 1974). It can be shown in the experimental model of diabetes mellitus induced by STZ, a beta-cytotoxic product of *Streptomyces achromogenes* (Brosky and Sogothopoulos 1971), by insulin treatment (Seyer-Hansen 1976) or renal transplantation (Lee et al. 1984) that this increase in kidney mass is not due to a direct action of the toxin, but is rather the consequence of the functional and metabolic alterations characterizing the diabetic state. Among these functional consequences, the increases in nephron plasma flow (Hostetter et al. 1981) glomerular filtration rate (Hostetter et al. 1981; Finn 1982) Na^+ , K^+ -ATPase activity (Wald and Popovtzer 1984, Ku et al. 1987) and glucose and Na^+ reabsorption (Harris et al. 1986; Seyer-Hansen 1987), and the induction of osmotic diuresis (Hostetter et al. 1981; Finn 1982) may be of relevance here. As a morphological correlation of these functional alterations, an increase in glomerular filtration surface area was found by stereologic investigations (Osterby et al. 1978, Krostrup et al. 1977).

Although the causative factors appear to be different, the growth effect in early experimental diabetes is similar to that observed in the classical experimental model of induced kidney growth, i.e. compensatory hypertrophy after unilateral nephrectomy. The present data are in agreement with those from the literature which show that the growth response is of the same order of magnitude in both diabetic and compensatory hypertrophy (Seyer-Hansen 1978). In contrast to the latter study, a significant additive effect on kidney growth was not observed in the DN animals in the present study. Such a discrepancy may be explained by the observations of Kunjara et al. (1986) showing that the additional growth response induced in diabetic animals by unilateral nephrectomy is more pronounced during the later stages of experimental diabetes.

The present quantitative ultrastructural data confirm earlier findings which showed that the volume and numerical densities of AVs are significantly decreased in the cortical convoluted tubular cells after unilateral nephrectomy (Jurilj and Pfeifer 1985). The additional finding that these parameters are also decreased in early

diabetic kidney growth and the fact that the decrease is of the same order of magnitude as in compensatory growth after unilateral nephrectomy can be taken as further support for the hypothesis that inhibition of cellular autophagy is an important factor in growth in several different parenchymal organs (for review see Pfeifer 1982).

As discussed in detail elsewhere, changes in the AV parameters are interpreted here as reflecting changes in the rate of AV formation and of the degradation of their contents, because alterations in the lifespan of AVs known to occur under the influence of various inhibitors are unlikely to be features of the present experiments. Furthermore the decrease in AV volume and numerical density cannot be interpreted merely as a "dilution" effect due to the increase in kidney mass, since this increase amounts to less than 10% at day 1, whereas the AVs are reduced at this time point by 50% or more.

Although a decrease in kidney AV volume and numerical density could be expected during renal growth, this finding appears to conflict with the known effects of insulin PTCs in the normal kidney. As reported earlier (Pfeifer and Wahrmuth-Metz 1983), the volume and numerical density decreases within a short time after both the systemic and local application of insulin, which is in line with the general anticatabolic effect of this hormone. Insulin deficiency as in STZ diabetes should, therefore, increase the AV parameters in the kidney. It appears that the still unclarified factors that are responsible for diabetic kidney growth are more relevant to the regulation of cellular autophagy than the isolated action of insulin on these tubular cells.

Among the factors discussed as being decisive in the induction of a growth response in the kidney, the increase in cellular metabolic work should be considered in some detail. The increased load on the nephron in early diabetes and after unilateral nephrectomy has been analyzed particularly with respect to the increase in tubular Na^+ reabsorption. However, the fact that the growth response, both after unilateral nephrectomy (Katz et al. 1978) and diabetic hypertrophy (Seyer-Hansen 1987) precedes the maximum Na^+ load appeared to contradict the work load hypothesis. It has also been shown that unilateral ureteroperitoneostomy, which increases the excretory work of the kidneys, does not stimulate growth to a significant extent (Gittes and Gittes 1984). However, since only a small proportion of the total metabolic work of the kidney is required for excretion, i.e. the production of urine, it would require an analysis of the total metabolic work to determine whether this could be a significant factor in the regulation of kidney growth. The fact that metabolic unloading of specialized parts of the nephron can be associated with stimulation of cellular autophagy, such as occurs in the thick ascending limb of Henle's loop under the influence of furosemide (Bahro et al. 1988), would be in line with the general assumption that work load inhibits cellular autophagy and by this means induces adaptive growth.

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