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Rapid development of glomerulosclerosis in diabetic Dahl salt-sensitive rats

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Summary Diabetic nephropathy tends to develop more readily in patients with a family history of hypertension and/or disturbances in sodium transport across the plasma membrane. This prompted us to study the renal effects of diabetes mellitus in a rat strain which is predisposed to develop salt-sensitive hypertension, the Dahl salt-sensitive rat. Diabetes is associated with several aberrations in the renal handling of sodium, such as elevation of tubular Na^+, K^+ATPase activity. This effect was more pronounced in Dahl salt-sensitive than in Dahl salt-resistant rats. Severe renal lesions, characteristic of the advanced phase of diabetic nephropathy are very rarely observed in rats with streptozotocin diabetes. However, 2 months after induction of diabetes, the Dahl salt-sensitive rats had morphological signs of advanced glomerular disease. The urinary albumin concentration was very high, but did not correlate with the blood pressure. Non-diabetic Dahl salt-sensitive rats as well as Dahl salt-resistant diabetic and non-diabetic rats had little or no signs of glomerular disease and consistently very low urinary albumin concentrations. [Diabetologia (1997) 40: 367–373]

Keywords Dahl rats, salt sensitivity, diabetic nephropathy.

For unidentified reasons, nephropathy develops only in a subset of insulin-dependent diabetic patients. At present there is no explanation why some patients develop diabetic nephropathy (DN) while others do not [1]. Although the cumulative incidence of DN has decreased substantially as a result of improved glycaemic control [2, 3], the susceptibility to DN cannot only be explained by differences in the metabolic state [4].

In humans, DN is commonly associated with hypertension. It is well-established that hypertension aggravates DN. There is also evidence that the genetic trait(s) for hypertension may predispose to diabetic renal disease. Non-diabetic parents of patients with DN have higher blood pressure levels than the average population [5]. The erythrocyte sodium-lithium counter transport activity, which is a genetic marker for essential hypertension in non-diabetic individuals [6] is increased in patients with overt DN compared to those without DN [7, 8]. This suggests that a predisposition to essential hypertension, with or without alteration in the regulation of electrolyte transport, contributes to susceptibility to DN.

Altered sodium metabolism is a consistent finding in diabetes, and it is present even in the absence of overt renal or cardiac disease. Both insulin-dependent and non-insulin-dependent diabetic patients have been reported to have a significant increase of total exchangeable sodium [9, 10]. Considerable attention has been focused on abnormalities of renal sodium handling in diabetes, since the kidney is the main regulator of body salt and water homeostasis. In a previous study [11] we demonstrated that the

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Abbreviations: DR, Dahl salt-resistant; DS, Dahl salt-sensitive; DRD, diabetic Dahl salt-resistant; DSD, diabetic Dahl salt-sensitive; GFR, glomerular filtration rate; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto; DN, diabetic nephropathy.

increased filtered load of glucose in diabetes will, via activation of the sodium/glucose co-transporter, stimulate proximal tubular Na^+, K^+ATPase activity and increase tubular sodium reabsorption.

Taken together, these observations have prompted us to study whether disturbances in the renal handling of salt and/or a genetic predisposition to hypertension promotes the development of DN. The Dahl salt-sensitive rat, which develops severe hypertension when fed a high salt diet, provides an excellent model for addressing these questions [12].

Materials and methods

Animals. The study was performed on male Dahl salt-sensitive (DS) and Dahl salt-resistant (DR) rats purchased from Møllegaards Breeding Centre (Ejby, Denmark). Principles of laboratory animal care as well as the specific Swedish national law were followed [13]. The rats were fed a standard rat chow (B&K Universal, Sollentuna, Sweden) containing 18% protein and 0.22% Na and provided tap water ad libitum. The rats were randomly allocated into control (DR and DS) and diabetic (DRD and DSD) groups at the age of 6–7 weeks. Half of the rats were made diabetic with streptozotocin (Zanosar, Upjohn Company, Kalamazoo, Michigan), 65–70 mg/kg, injected into the tail vein. Blood glucose concentration was measured regularly, and streptozotocin-treated rats with non-fasting plasma glucose concentration below 16.5 mmol/l at 48 h after injection were excluded from the study.

Short-term studies

Na^+, K^+ATPase activity. Na^+, K^+ATPase activity was measured 10–12 days after the induction of diabetes in the diabetic rats and at the corresponding age in non-diabetic animals. The rats were anaesthetized with Inactin-Byk (Byk-Gulden, Konstanz, Germany) 80 mg/kg body weight i.p. Kidney perfusion and tubule dissection were performed as described [14]. Briefly, the left kidney was perfused with a modified Hank's solution (in mmol/l): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 1 $CaCl_2$, 1 $MgCl_2$, 10 Tris (hydroxymethyl) aminomethane hydrochloride (Tris HCl), containing 0.05% collagenase (Sigma, St. Louis, MO, USA) and 0.1% bovine serum albumin (Behringwerke, Marburg, Germany). The kidney was removed and cut along its corticopapillary axis into small pieces, incubated at 35°C for 20 min in 10 ml perfusion solution with 10^{-3} mol/l butyrate, bubbled with oxygen. The tissue was then rinsed and transferred to the same solution as the perfusion solution, but without collagenase and bovine serum albumin, and with a $CaCl_2$ concentration reduced to 0.25 mmol/l. In order to optimise mitochondrial respiration, butyrate (10^{-3}) was also added to the solution. Proximal tubules were then dissected manually from the outer cortex and individually transferred to the concavity of a bacteriological slide and photographed for length determination in an inverted microscope at × 100. The tubules were stored on ice while dissection was finished after at most 30 min.

Determination of Na^+, K^+ATPase activity. Na^+, K^+ATPase activity was measured in single proximal tubules. The tubule segments were made permeable by hypotonic shock, freezing and thawing. Individual segments were incubated for 15 min at 37°C in a medium containing the following (in mmol/l): 50 NaCl, 5 KCl, 10 $MgCl_2$, 1 EGTA, 100 Tris-HCl, 10 Na_2ATP (grade II; Sigma, St. Louis, MO, USA) and [γ-^{32}P] ATP (New England Nuclear, Boston, Mass., USA) 2–5 Ci mmol/l^{-1} in tracer amounts (5 nCi μl^{-1}). For determination of ouabain-insensitive (Mg-dependent) ATPase activity, NaCl and KCl were omitted, Tris HCl was 150 mmol/l, and 2 mmol/l ouabain (Merck, Darmstadt, Germany) was added. The pH of both solutions was 7.4. The phosphate liberated by hydrolysis of [γ-^{32}P]ATP was separated by filtration through a Millipore filter after the absorption of unhydrolysed nucleotide on activated charcoal. The radioactivity was measured in a liquid scintillation spectrophotometer. In each study, we determined total ATPase activity and Mg-ATPase activity in six to eight segments. The phosphate released from [γ-^{32}P]ATP in six to eight samples of incubation solution containing no tubule segments was determined to correct for non-specific ATP hydrolysis. Na^+, K^+ATPase activity was calculated as the difference between the mean values for total ATPase activity and Mg-ATPase activity.

Long-term studies

Renal function. In separate rats, 6–8 weeks after the induction of diabetes in the diabetic animals, and in the corresponding age of non-diabetic rats glomerular filtration rate (GFR) was determined by the clearance of inulin (Inutest; Laevosan Gesellschaft, Linz, Austria). Inulin was diluted in normal saline (5%), and infused at a rate of 1 ml · 100 g body weight^{-1} · h^{-1}. The infusion was preceded by a priming dose of the infusate, 1 ml/100 g body weight. After 60 min equilibration time urine was collected from tubes placed in both ureters and sampled for two (45–60 min) periods [15]. Tubular sodium reabsorption was calculated from the filtered and excreted sodium measured during the clearance study. Chemical analysis of inulin and sodium in blood and urine was performed by standard laboratory method [16].

Albuminuria, mean arterial pressure. During the clearance study urine was collected for the examination of albuminuria. Urinary albumin concentration was determined on a MIRA auto-analyzer (COBAS, Japan) using immunoturbidometry, in the samples collected during the clearance studies. Mean arterial pressure was recorded via one carotid artery as described [17]. The kidneys were then removed for morphometric studies.

Renal morphology. Morphometric studies were assessed 6–8 weeks after the induction of diabetes. The time course for the development of glomerulosclerotic lesions has been studied in a separate set of experiments. The kidneys (left kidney) were immersion fixed in 4% paraformaldehyde, and cut in 1.5 mm thick coronal slices, from which a set of three systematically random slices was collected and embedded in paraffin. Sections (3 μm thick) were stained with periodic acid Schiff. For electron microscopic examinations cortical tissue was fixed in 3.0% glutaraldehyde and embedded in Epon. Ultrathin sections were stained with uranylacetate and lead citrate and studied in a JEM 100S electron microscope (Philips, The Netherlands). In pilot studies, light microscopy revealed distinctive glomerular changes in the diabetic DS rats compared to all other groups, consisting of mesangial expansion due to an increase of the matrix substance and segmental as well as global sclerosis. No obvious increase in the number of mesangial cells per mesangial area was found by light or electron microscopy.

Light microscopy. Estimation of the glomerular mesangial expansion was performed by light microscopy by point counting using a point ocular grid with 19.87 mm between each point at tissue level (× 420). Since the DR and DRD rats did not present any glomerulosclerotic lesions, glomeruli from those two groups were selected systematically from one section level of tissue blocks. Approximately 40 glomeruli from each rat were counted. In the DS and DSD rats serial sectioning was performed. Every third section was collected and in the midst of a set of 30 sections per block each selected glomerulus was followed throughout, up and down in each section. Only glomeruli free from sclerotic lesions were included. Thus, 20 glomeruli per animal were analysed. The volume fraction of the mesangium per glomerulus (V/v mesangium per glomerulus) was estimated from the total number of points hitting the mesangial area divided by the total number of points hitting the whole tuft. Sclerotic lesions were defined as collapsed, obliterated capillaries with sparseness of normal cellular elements. The percentage of segmentally and globally sclerotic glomeruli was counted out of 150 glomeruli in one section level of the tissue blocks from all the different animal groups.

Electron microscopy. The V/v mesangium and V/v matrix per glomerulus were determined in DS and DSD rats by electron microscopy. The tissue blocks were cut into 1-μm thick sections. At 10-μm intervals toluidine blue stained sections were inspected to avoid sampling glomeruli with sclerotic lesions. The sections were sequentially cut at 30-μm levels, and glomerular profiles from glomeruli that had been followed throughout the whole capillary tuft and that had not shown any sclerotic lesions were selected. Four glomerular profiles per animal were analysed. We took 17–18 electron micrographs, covering approximately 50 % of the total area of each glomerulus at × 15000 magnification. The V/v mesangium and V/v matrix per glomerulus were analysed by point counting using a superimposed lattice square grid with points 30 μm apart. The same definitions for the reference volume of the glomerular tuft and the geographical definition of the mesangium and its delineation to the peripheral capillary were applied as stated previously [18].

Statistical analysis

Values are expressed as mean ± SEM, unless otherwise stated. Since urinary albumin concentration has a skewed distribution, values were log transformed. The statistical analysis was performed with the Student's *t*-test and with analysis of variance (ANOVA), followed by a multiple comparison procedure (Tukey-Kramer's HSD test) when appropriate. *P* values less than 0.05 were considered significant.

Results

Na^+, K^+ATPase activity in proximal tubules. Two weeks after the induction of diabetes all diabetic rats had significantly elevated blood glucose levels compared to the non-diabetic animals (diabetic rats vs control rats: 26.8 ± 1.7 vs 6.5 ± 0.9 mmol/l, $p < 0.001$). Na^+, K^+ATPase activity was similar in non-diabetic DS and DR rats (DR: 2269 ± 71 pmol P_i mm tubule^{-1} h^{-1}, $n = 7$; DS: 2181 ± 64 pmol P_i mm tubule^{-1} h^{-1}, $n = 8$) (Fig. 1). Diabetes significantly increased Na^+, K^+ATPase activity both in DR and DS groups compared to the non-diabetic animals, but in DSD rats N^+, K^+ATPase activity was significantly higher (4501 ± 263 pmol P_i mm tubule^{-1} h^{-1}, $n = 6$) than in DRD rats (3772 ± 132 pmol P_i mm tubule^{-1} h^{-1}, $n = 5$).

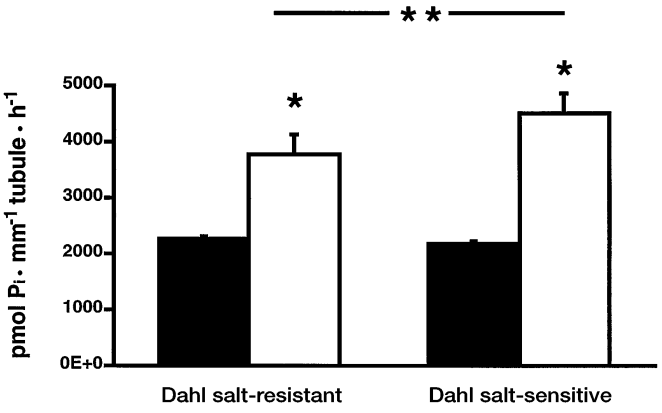

Fig. 1. Na^+, K^+, ATPase activity measured as ouabain-sensitive ATP hydrolysis in single proximal tubule segments of the different animal groups. Each value represents the mean ± SEM of 5–8 animals in each experiment, in each of which ATPase activity was determined in 6–8 tubule segments. ■, Non-diabetic rats; □ diabetic rats. * $p < 0.05$ compared to the non-diabetic rats; ** $p < 0.001$ between DRD and DSD rats

Table 1. Urinary albumin concentration (U_{alb}) and mean arterial pressure (MAP) in different groups of rats 6–8 weeks after the induction of diabetes (long-term studies)

	U_{alb} (mg/ml) median; range	MAP (mm Hg) mean ± SEM
Dahl salt-resistant $n = 6$	6.96 2.77–17.5	126.9 ± 4.5
Dahl salt-sensitive $n = 8$	< 1.69	146.9[b] ± 7.8
Diabetic Dahl salt-resistant $n = 6$	3.94 1.19–13.03	125.0 ± 6.2
Diabetic Dahl salt-sensitive $n = 6$	131.8[a] 56.0–309.0	156.0[b] ± 6.8

[a] $p < 0.001$ compared to all other groups; [b] $p < 0.05$ compared to DR and DRD

Microalbuminuria and mean arterial pressure. At 6–8 weeks after the induction of diabetes, urinary albumin concentration was very low in all non-diabetic rats (Table 1). The slight difference between the two non-diabetic groups was negligible. Diabetes did not provoke microalbuminuria in DRD rats even after 2 months of sustained hyperglycaemia. Although blood pressure was comparable in the DS and DSD rats, microalbuminuria only occurred in the diabetic DSD rats (Table 1). In the DSD group, significant increase in the degree of albuminuria developed after

Table 2. Morphometric analysis by light microscopy in different groups of rats

Group	Mesangium/ glomerulus (Vv%)	Segmental sclerosis (%)	Global sclerosis (%)
Dahl salt-resistant $n = 8$	15.89 ± 0.35	0	0
Diabetic Dahl salt-resistant $n = 7$	16.33 ± 0.37	0	0
Dahl salt-sensitive $n = 8$	18.28 ± 0.35	6.37 ± 0.70	0
Diabetic Dahl salt-sensitive $n = 6$	28.75[a] ± 1.17	10.03[b] ± 0.97	0.77 ± 0.41

Data are mean ± SD
[a] $p < 0.001$ compared to all other groups; [b] $p < 0.01$ compared to Dahl salt-sensitive

Table 3. Morphometric analysis by electron microscopy in different groups of Dahl salt-sensitive rats

Group	Mesangium/ glomerulus (Vv%)	Matrix/ glomerulus (V/v%)
Dahl salt-sensitive $n = 8$ coefficient of variation	17.46 ± 0.4 0.13	6.20 ± 0.21 0.15
Diabetic Dahl salt-sensitive $n = 6$ coefficient of variation	29.53[a] ± 1.75 0.14	13.07[a] ± 0.47 0.15

Data are mean ± SD
[a] $p < 0.001$ compared to Dahl salt sensitive rats

week 3 of diabetes, since urinary albumin concentration was similarly low in DSD (2.28; 1.81–2.86 mg/ml and DRD (6.0 mg/ml) rats after 3 weeks' diabetes duration. At this time point, i. e. 3 weeks after the induction of diabetes, the diabetic groups had somewhat higher blood pressure than the non-diabetic animals [DR: 112.0 ± 3.0, DS: 114.2 ± 4.0, DRD: 130.4 ± 4.8, DSD: 135.0 ± 7.7 mm Hg. The difference between the diabetic and non-diabetic groups was significant ($p < 0.05$).] Diabetes did not provoke further elevation in the blood pressure in the salt resistant animals, and in the long-term studies blood pressure was comparable and significantly lower in the DR and DRD rats than in the DS and DSD groups (Table 1). Significant microalbuminuria occurring in the diabetic DSD rats could not be attributed to the slightly elevated blood pressure, since urinary albumin concentration was negligible in the DS rats with almost identical blood pressure values (Table 1). Furthermore, the degree of albuminuria did not correlate with the blood pressure within the salt-sensitive groups (data not shown).

Renal function. Glomerular filtration rate (GFR) was comparable in DR (1.54 ± 0.13 ml/min) and DS (1.59 ± 0.13 ml/min) rats. GFR in the diabetic DRD rats (3.35 ± 0.41 ml/min) was significantly elevated ($p < 0.001$) compared to the non-diabetic animals. Although diabetic DSD rats also exhibited significantly ($p < 0.001$) elevated GFR (2.65 ± 0.14 ml/min) compared to the non-diabetic rats, GFR in these rats had already started to decline, and reached a level which was significantly lower ($p < 0.05$) than GFR in the diabetic DRD rats.

Tubular sodium reabsorption was significantly higher in the diabetic rats (DRD: 460.5 ± 43.6; DSD: 385.2 ± 20.5 μmol/min) compared to the non-diabetic animals (DR: 217.2 ± 17.4; DS: 237.1 ± 14.6 μmol/min. The difference between DSD vs DS and DR $p < 0.01$; DRD vs DR and DS, $p < 0.001$.)

Renal morphology. At 6–8 weeks after the induction of diabetes DSD rats demonstrated pronounced glomerular lesions with an increase in mesangial matrix, mesangial widening and focal segmental or focal global glomerulosclerosis (Figs. 2 and 3; Table 2 and 3). The results from a morphometric evaluation of the glomeruli in DS and DR rats based on light microscopy are depicted in Table 2. Significant differences between DSD and all other groups were found with regard to mesangial expansion and to the occurrence of segmental and global glomerular sclerosis. According to the morphometric evaluation by electron microscopy, mesangial expansion was due to enhancement of the mesangial matrix (Table 3). A time course study on the structural changes in DSD rats has shown that significant mesangial matrix expansion (V/v mesangium/glomerulus) occurred already between the first and third week (16.7 ± 0.6 vs 24.4 ± 0.6 %, $n = 3$, $p < 0.001$). In the non-diabetic DS rats, some segmentally sclerotic glomeruli were also seen, but no increase in mesangial matrix was noted. Glomerulosclerosis was not detected in DRD and DR rats. In addition, the DSD rats often showed hyaline deposits in the glomeruli. Even if the latter changes were more pronounced in glomeruli with sclerotic lesions, they were also obvious in glomeruli free of sclerosis. Tubulo-interstitial changes consisted of a mild focal tubular atrophy and fibrosis. Such changes were minimal in the non-diabetic DS rats. No obvious medial or intimal thickening of arterial and arteriolar vessels was noted in the different groups; however, discrete hyaline deposits were detected in the arterial vessels of DSD rats.

Discussion

The structural changes in the kidney, which occur early in the course of diabetes, are very similar in the rat and human [19]. However, morphological signs of more advanced diabetic nephropathy, like increases in mesangial matrix, do not generally progress in the

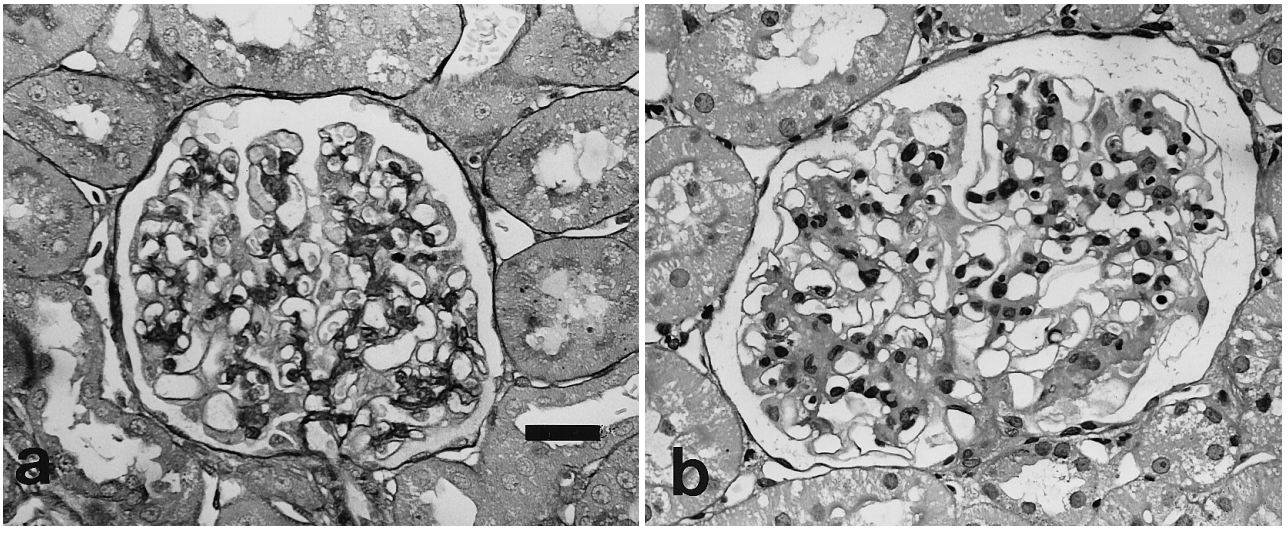

Fig. 2. a, b. Light microscopic sections of representative glomerular profiles from DRD (**a**) and DSD (**b**) rats. Clearly expanded mesangial areas are present in **b** compared with **a**. The bar (**a**) represents 40 μm. Periodic acid schiff staining

rat to the stage comparable with end-stage human glomerulopathy [20]. Mesangial expansion accompanied by focal segmental/global sclerosis has been observed in non-diabetic hypertensive DS rats, but only after a long period of time (12 and 28 weeks, respectively) and if simultaneously challenged by a high salt diet [21, 22]. Part of the increase in mesangial volume in the latter model [22] might be attributed to age-related changes [20]. However, in our model, diabetes induced in DS rats resulted in a rapid development of glomerulopathy mostly characterised by mesangial expansion typical of the advanced phase of diabetic nephropathy.

The relationship between hypertension and changes in kidney function and morphology in rats with streptozotocin-induced diabetes has previously been evaluated in two studies on spontaneously hypertensive rats (SHR) [23, 24]. The SHR rats are considered to be salt-insensitive. Normotensive, salt-insensitive Wistar Kyoto rats (WKY) were used as their controls. In both studies the SHR rats were hypertensive compared to the WKY rats. In one study [23] no difference was found between SHR and WKY rats with regard to the development of diabetic nephropathy. In the other study [24] the rats were followed for 8 months after the induction of diabetes. The blood pressure in these SHR rats was higher than in our DSD rats. There was little difference in the renal morphological alterations between the diabetic and non-diabetic SHR rats. Despite the long follow-up time, the changes in the diabetic SHR rats appeared, according to the description, to be less pronounced than in our DSD rats. These previous studies strongly support the concept that the rapid development of glomerular morphological changes, compatible with the appearance of diabetic nephropathy, in the DSD rats, cannot only be attributed to their moderate hypertension, but may also be related to their salt-sensitivity as such.

Hyperglycaemia is the most important cause of vascular complications in diabetes [25]. The cellular mechanisms of action are not yet elucidated, but are most likely multiple [26]. The glomerular lesions and hypertension may well be related to the sodium retention induced by hyperglycaemia. We have previously reported on the causal relationship between hyperglycaemia and sodium retention [11]. The DS rats which have been described as the animal model most closely resembling human hypertension [27], have an altered sodium metabolism that also predisposes to sodium retention, extracellular volume expansion and hypertension [12, 28, 29]. Diabetes increases proximal tubular Na^+, K^+ATPase activity in all species and rat strains studied [30–34]. High glucose concentration in the tubular fluid is mainly responsible for this increase [11]. Na^+, K^+ATPase is responsible for the active transport of sodium across the tubular cell. Diabetes resulted in a more pronounced increase in Na^+, K^+ATPase activity in the DSD than in the DRD rats. Since high tubular Na^+, K^+ATPase activity in DSD rats can aggravate sodium retention it may also contribute to the rapid development of nephropathy in the diabetic DS rats.

The relationship between early functional abnormalities and late structural alterations in the complex aetiology of diabetic nephropathy remains to be fully determined. The glomerular morphological changes in our study correlated more with the state of diabetes than with the actual level of blood pressure. This finding further supports the concept that diabetes induced in animals with a genetic trait for salt-sensitive hypertension accelerates the development of diabetic nephropathy.

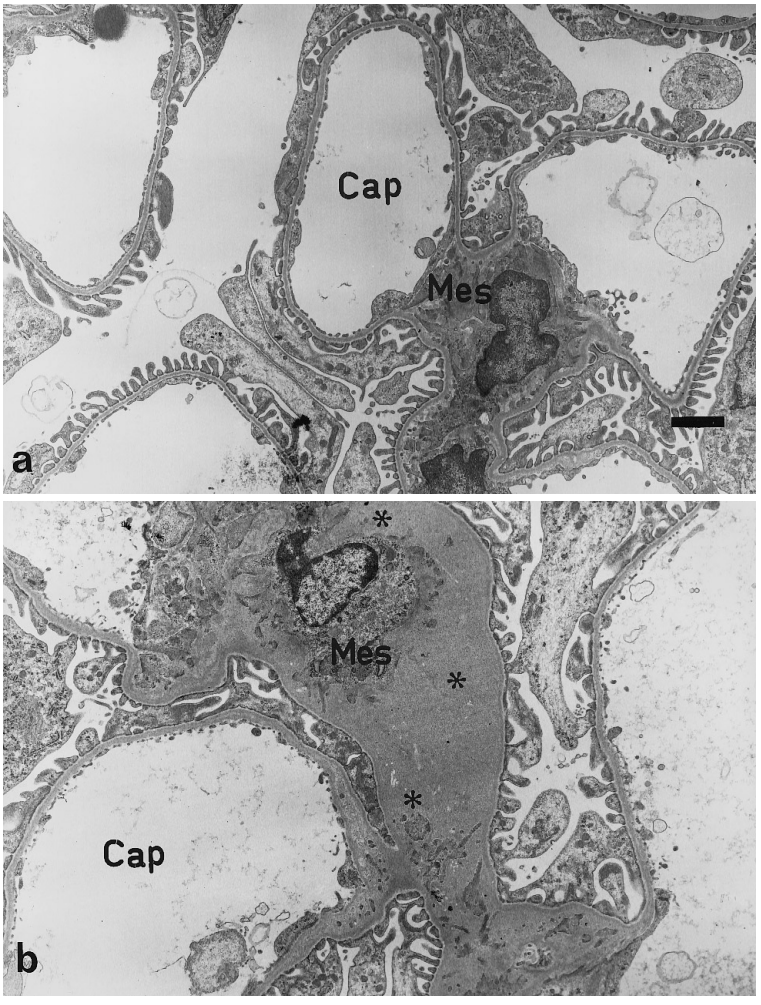

Fig. 3. a, b. Electron micrographs of glomerular capillary segments from DRD and DSD rats (**a** and **b**). In **a** the mesangium shows no apparent changes compared with **b,** where the mesangium is expanded due to an augmentation of the matrix substance (*). The bar (**a**) represents 1 μm. Mes, Mesangium, Cap, capillary lumen

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