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Ischemia causes rapidly progressive nephropathy in the diabetic rat

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Ischemia causes rapidly progressive nephropathy in the diabetic rat. We examined the influence of renal ischemia in rats with diabetes mellitus (DM). Male Wistar rats were rendered diabetic by streptozotocin treatment. Two weeks later, 30 minutes of complete ischemia was induced in the left kidney of DM and non-DM animals. Both groups were evaluated functionally and morphologically four or eight weeks post-ischemia. In non-DM animals renal function and morphology showed almost complete recovery. In the DM animals, however, this comparatively short period of ischemia caused a substantial loss of renal function. Four weeks post-ischemia inulin clearance in the DM kidneys rendered ischemic was only 20% of that in the corresponding non-DM kidneys, and after eight weeks the DM kidneys were completely anuric. Extensive inflammation and tubulointerstitial fibrosis were evident in DM kidneys four weeks after ischemia and seemed to increase over time. After eight weeks, tubular atrophy was found in the ischemic DM kidneys, resulting in a substantial loss of kidney mass. We conclude that in diabetic rats renal ischemia causes rapidly progressive kidney damage that in several respects resembles diabetic nephropathy in humans. Since advanced renal lesions similar to those seen in human diabetic nephropathy never develop in the rat solely as a result of DM, the present study may provide an experimental model for further studies on renal failure in diabetes mellitus.

In experimental rodent models of diabetes mellitus (DM), advanced lesions similar to those seen in human diabetic nephropathy never develop. Only early signs of diabetic renal injury, such as mesangial cell expansion and basal membrane thickening, are found [1–5]. Furthermore, these early lesions can be reversed by insulin treatment [6]. The lack of severe DM nephropathy in the rat may be due to the short lifespan, in that there is not enough time to develop the complete syndrome. On the other hand, the early diabetic lesions develop rapidly in experimental rodent models. It seems possible that more severe damage, corresponding to the lesions in human diabetic nephropathy, requires other factors for its development in addition to insulin deficiency and hyperglycemia. One such possible factor is ischemia.

More than 30 years ago Thomsen suggested that some of the pathological findings in diabetic nephropathy, in particular the interstitial inflammation and fibrosis, may be explained by ischemia [7]. It is now generally accepted that ischemia-reperfusion injury causes inflammation, and diabetes has recently been found to exacerbate this inflammatory response [8]. It has been suggested that the tubulointerstitial fibrosis may be mediated by inflammatory cells [9] and correlate with the degree of arterial obliteration [10].

Only in a few studies has the combination of renal ischemia and DM been assessed. Wald et al found that whereas DM rats were more sensitive to ischemia [11], they were protected against nephrotoxic acute tubular necrosis [11, 12]. Kuramochi and Homma observed an abnormal decrease and recovery in oxygen consumption in kidney slices from diabetic rats after ischemia [13, 14]. There are indications that an impaired response to nitric oxide might lead to a more pronounced decrease in renal function in the early post-ischemic phase in DM rats [15].

All these previous studies have concerned the early phase of ischemic renal failure in the diabetic rat. In the present study we investigated the long-term effect of transient renal ischemia in rats previously made diabetic by administration of streptozotocin (STZ). We used an ischemic period of 30 minutes, which in non-DM animals results in almost complete recovery. In the diabetic rats, however, both functional and morphological evaluation revealed rapidly progressive renal damage. Thus, end-stage renal failure was attained within eight weeks.

METHODS

Experimental groups and animals

Male Wistar rats (Møllegaard, Denmark) were used. They were divided into four groups, of which two were subjected to diabetic induction by STZ and two were used as controls. Unilateral ischemia was induced in all groups. The rats of one DM and one non-DM group were examined four weeks after ischemia, and those of the other two groups were studied eight weeks after ischemia. For the entire duration of the experiments the animals were kept in plastic cages and fed standard rat chow (R36; Lactamin AB, Stockholm, Sweden) and water *ad libitum*.

Induction of diabetes

Before induction of DM the rats were deprived of food overnight but had free access to water. Under light ether anesthesia, a solution containing 11 mg/ml of STZ (Zanosar; Upjohn, Kalamazoo, MI, USA) in saline with a sodium citrate buffer, pH 4.0, was injected into the femoral vein. The dose of STZ given was

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Table 1. Metabolic parameters

	Non-diabetic		Diabetic	
	4 weeks (N = 8)	8 weeks (N = 8)	4 weeks (N = 7)	8 weeks (N = 8)
Blood glucose *mmol/liter*	10.7 ± 1.1	10.1 ± 0.5	31.2 ± 0.9[b]	28.1 ± 1.4[b]
Water consumption *ml/day*	29 ± 2	29 ± 4	182 ± 17[b]	157 ± 13[b]
Body weight *g*	294 ± 13	365 ± 15	240 ± 14	291 ± 10[a]

Values are means ± SEM. Diabetic animals are compared with non-diabetic animals observed for the same time.
[a] $P < 0.01$ DM vs. non-DM
[b] $P < 0.001$ DM vs. non-DM

Table 2. Renal function

		Non-diabetic		Diabetic	
Observation time		4 weeks (N = 8)	8 weeks (N = 8)	4 weeks (N = 7)	8 weeks (N = 8)
Urinary flow *µl/min*	Ischemic	6.1 ± 1.5	3.8 ± 1.1	4.2 ± 3.5	0[c]
	Non-ischemic	7.6 ± 2.1	5.7 ± 1.6	33.7 ± 9.7[a]	47.4 ± 4.7[c]
C_{In} *ml/min*	Ischemic	0.75 ± 0.15	0.55 ± 0.16	0.14 ± 0.13[b]	0[b]
	Non-ischemic	0.86 ± 0.12	0.76 ± 0.18	1.44 ± 0.38	1.85 ± 0.16[b]
Hct %		52 ± 1	54 ± 2	43 ± 3[d]	47 ± 2
Arterial blood pressure	*mmHg*	73 ± 5	64 ± 4	69 ± 5	67 ± 5

Values are means ± SEM. The arterial blood pressures shown are from when the blood sample for inulin determination was drawn.
[a] $P < 0.05$ DM vs. non-DM in corresponding kidneys
[b] $P < 0.01$ DM vs. non-DM in corresponding kidneys
[c] $P < 0.001$ DM vs. non-DM in corresponding kidneys
[d] $P < 0.05$ DM vs. non-DM groups

55 mg/kg body wt. The food and water consumption, weight gain and blood glucose levels were recorded to monitor the degree of diabetes. Blood glucose was assayed by the glucose-dehydrogenase method (Granutest 250; Merck, Whitehouse Station, NJ, USA). At least two weeks were allowed to elapse between the induction of diabetes and the ischemic injury. The rats were considered diabetic if the blood glucose level was above 20 mmol/liter.

Induction of ischemia

The animals were anesthetized by an intraperitoneal injection of Eqviticin (a mixture of chloral hydrate 183 mg · kg^{-1} body wt, magnesium sulfate 0.09 mg · kg^{-1} body wt and thiobarbital sodium 42 mg · kg^{-1} body wt; Apoteksbolaget, Umeå, Sweden). During the operation the animals were placed on a servocontrolled heating pad, which kept the body temperature at 37.5°C. A midline incision was made and the left renal artery was located and dissected free from its surrounding structures. After a recovery period of 10 minutes, renal ischemia was evoked by clamping the left renal artery for 30 minutes. Subsequently the abdomen was sutured and the animals were returned to their cages.

Measurement of renal function

GFR was estimated from the clearance of inulin (C_{In}). The animals were anesthetized and put on an electric heating pad, as described above, and tracheostomized. Catheters were inserted into both ureters through a supravesical incision. The right femoral artery was catheterized for blood pressure monitoring and withdrawal of blood samples, and the femoral vein for infusion of saline (5 ml · kg^{-1} body wt · hr^{-1} in non-DM rats, and 10 ml · kg^{-1} body wt · hr^{-1} in DM rats); this solution also contained 5 mCi · ml^{-1} ^{3}H-inulin. After a bolus injection of 1 ml, an equilibration period of 45 minutes was allowed to elapse. Urine was then collected separately from both kidneys for 30 minutes. In the middle of this period a reference blood sample was drawn. The ^{3}H activity in the plasma and urine was measured by the liquid scintillation technique.

Histological evaluation

After completion of the functional studies, both kidneys were removed and the upper third of each kidney was put into a buffered 4% formalin fixation solution with 1% N-cetylpyridinium chloride and processed for histological examination. The sections were subjected to periodic acid-Schiff staining for detection of basal membrane and cell nuclei and picro-Sirius staining for investigation of fibrosis. The histological findings were blindly evaluated on coded samples by two investigators together. On the arbitrary scale used, 0 indicated no sign of inflammation, tubular dilation or fibrosis, 1 indicated mild changes, 2 moderate damage, and 3 severe lesions.

In order to evaluate the degree of tubular atrophy and/or glomerular injury, the number of glomeruli in four randomly-selected visual fields (magnification ×160) in the cortex of each kidney was counted. To estimate the relative glomerular size, two photographs were taken of the cortex on sections from each kidney. The sizes were then estimated by weighing the glomeruli cut out from the photographs. The average size of the glomeruli in the non-ischemic, non-DM group observed for four weeks was put at 1 and the values in the other groups were normalized to this value.

Statistical analysis

All data are presented as means ± SEM. A one-way ANOVA test was combined with Scheffe's post hoc test to compare physiological and metabolic variables between the experimental

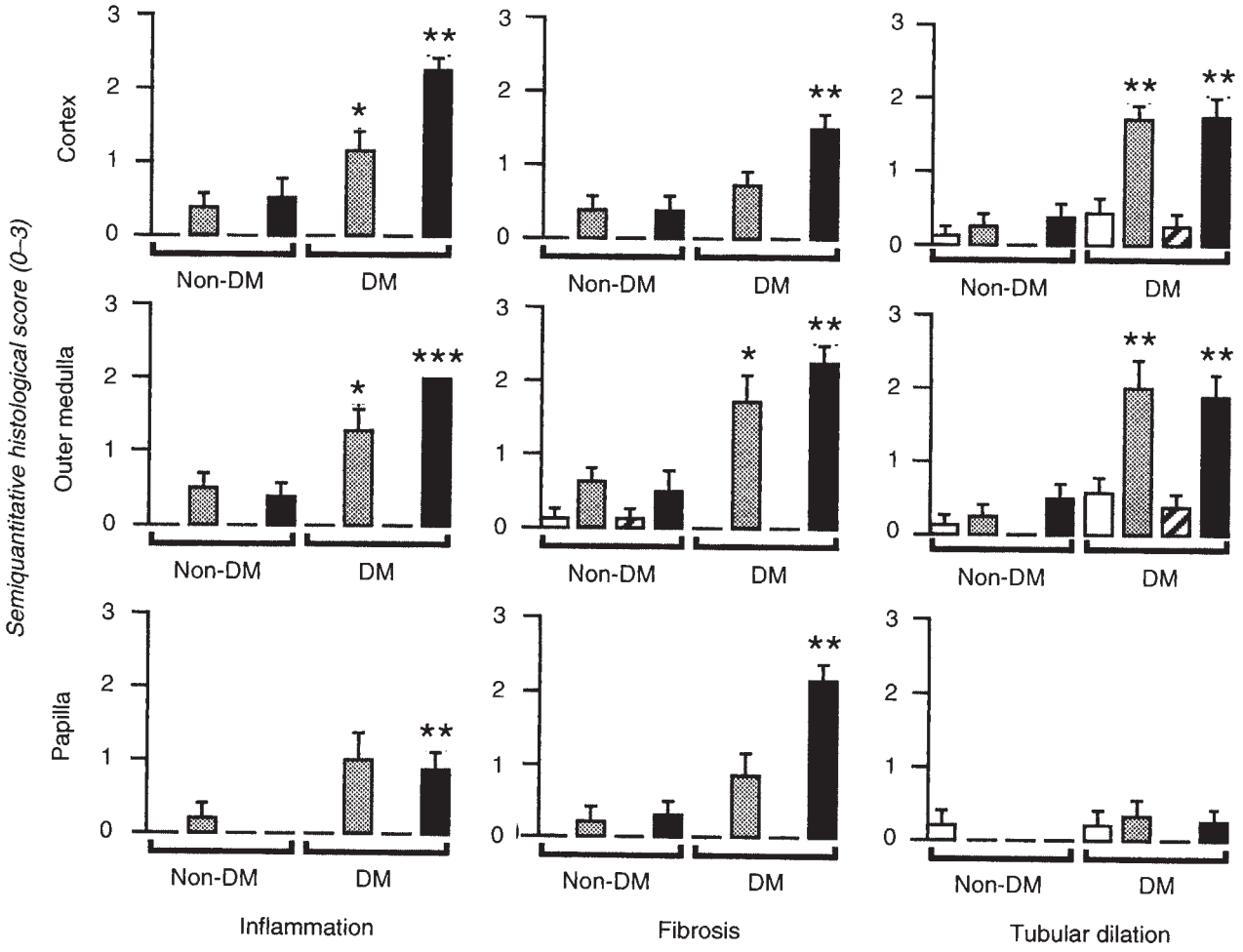

Fig. 1. Semiquantitative morphological evaluation of renal lesions. The number of animals is 7 or 8 except in papilla where it is 7 to 5. Symbols are: (□) 4 weeks non-ischemic; (▨) 4 weeks ischemic; (▨) 8 weeks non-ischemic; (■) 8 weeks ischemic. On the arbitrary scale used 0 indicated no sign of inflammation, tubular dilation or fibrosis, 1 indicated mild changes, 2 moderate damage, and 3 severe lesions. All values are means ± SEM. $^{*}P < 0.05$ DM versus non-DM in corresponding kidneys, $^{**}P < 0.01$ DM versus non-DM in corresponding kidneys and $^{***}P < 0.001$ DM versus non-DM in corresponding kidneys. Note that when no bar is shown the value is zero.

groups. The Mann-Whitney *U*-test was used for statistical comparisons of renal function values, number of glomeruli per visual field and semiquantitative morphological values between DM and corresponding non-DM kidneys from animals observed from the same length of time. Relative glomerular size was evaluated by pooling all relative glomerular sizes in one group and comparing corresponding kidneys in non-DM and DM groups using a one-way ANOVA test combined with Scheffe's post hoc test. For statistical analyses the Statview 4.5 program (Adobe Concepts, Berkeley, CA, USA) was used.

RESULTS

In Table 1 variables reflecting the degree of diabetes, that is, blood glucose levels, water consumption and body weight gain, are shown. It was evident that the STZ-treated animals were severely diabetic, and they drank about five times more water than the non-DM group. In spite of an increased food consumption the DM animals gained considerably less weight than the non-DM animals, probably due to loss of glucose in the urine. The mean blood glucose level was 30 mmol/liter in the DM and in the non-DM groups about 10 mmol/liter. Ketonuria was not present, however, indicating that some insulin production was spared.

In the DM animals, ischemia caused a substantial decrease in C_{In} to less than 20% of that in the non-DM post-ischemic kidneys after four weeks. This dramatic loss of renal function led to complete anuria within eight weeks post-ischemia. A compensatory increase in GFR in the contralateral non-ischemic kidney was found in the DM animals (Table 2).

Morphology

Hypertrophy of the non-ischemic DM kidneys was evident from a substantial increase in kidney size both four and eight weeks after the ischemic insult on the contralateral kidney. In the post-ischemic DM kidneys, however, no increase in size was seen after four weeks, and after eight weeks a substantial decrease in size was noted. Marked scarring was observed on the kidney surface four weeks post-ischemia, and this was even more pronounced after eight weeks.

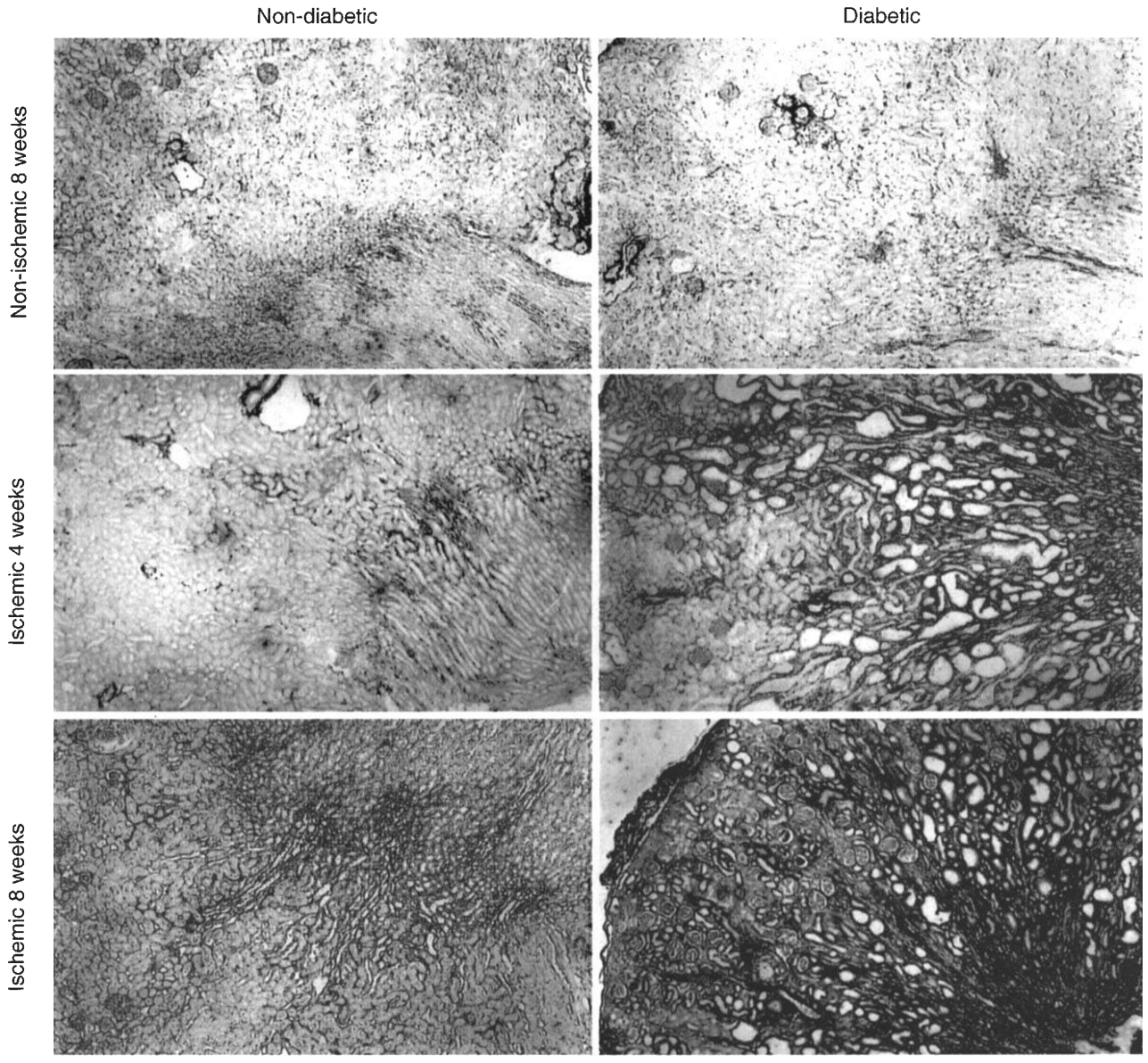

Fig. 2. Photomicrographs of Picro-sirius staining showing fibrosis. Severe fibrosis could be noted in the DM-kidneys subjected to ischemia especially after eight weeks. In the non-DM ischemic groups only small signs of fibrosis were present. In the DM kidneys subjected to ischemia pronounced tubular dilation was evident. Decrease in size and in the relative number of glomeruli in the cortex was obvious eight weeks post-ischemia due to tubular atrophy. Magnification ×70.

In the DM kidneys subjected to ischemia, infiltration of inflammatory cells consisting chiefly in macrophages and small lymphocytes, was found in all renal compartments except the glomeruli (Fig. 1). The inflammation was extensive both in the cortex and in the outer medulla and seemed to increase over time. In the papilla inflammatory cells were less frequent. In non-diabetic post-ischemic kidneys inflammatory cells were found only occasionally.

In the post-ischemic DM kidneys extensive fibrosis was observed both in the cortex and outer medulla (Figs. 1 and 2). Large areas of these kidneys were fibrotic and filled with inflammatory cells, and in these areas tubular structures could not be clearly identified. Eight weeks after the injury there was a marked increase in the number of glomeruli per visual field in the DM kidneys subjected to ischemia, indicating substantial tubular atrophy (Table 3). Tubular casts were only occasionally seen. In all DM kidneys subjected to ischemia pronounced tubular dilation was evident (Figs. 1, 2 and 3).

In the post-ischemic DM kidneys the glomeruli appeared heterogeneous. At four weeks post-ischemia some glomeruli were atrophic and sclerotic but most of them appeared almost normal, apart from thickening of Bowman's capsule (Fig. 3). Eight weeks after the ischemic insult the number of small and sclerotic glomeruli had increased. The relative glomerular size in the non-ischemic DM kidneys had increased by roughly one third after four weeks and by about two thirds after eight weeks (Table 3). In contrast, the glomeruli of the DM kidneys subjected to

Table 3. Relative glomerular size and number per visual field

		Non-diabetic		Diabetic	
		4 weeks (N = 8 rats)	8 weeks (N = 8 rats)	4 weeks (N = 7 rats)	8 weeks (N = 8 rats)
Relative glomerular size	Non-ischemic	1.00 ± 0.04	1.13 ± 0.03	1.37 ± 0.04[c]	1.69 ± 0.05[c]
	N glomeruli	100	91	66	75
	Ischemic	1.05 ± 0.04	1.26 ± 0.05	0.95 ± 0.04	0.85 ± 0.02[c]
	N glomeruli	90	89	88	177
Number of glomeruli per visual field	Non-ischemic	4.4 ± 0.4	4.1 ± 0.2	3.1 ± 0.1[a]	3.1 ± 0.3[a]
	Ischemic	3.4 ± 0.3	5.2 ± 0.4	4.1 ± 0.7	11.4 ± 1.9[b]

All values are means ± SEM. N is number.
[a] $P < 0.05$ DM vs. non DM in corresponding kidneys
[b] $P < 0.01$ DM vs. non DM in corresponding kidneys
[c] $P < 0.001$ DM vs. non-DM in corresponding kidneys

ischemia showed no hypertrophy at four weeks, and after eight weeks they were decreased in size.

DISCUSSION

The utility of experimental rodent models as a reflection of human diabetic nephropathy is limited, since advanced lesions never develop in the diabetic rat. In the present study we have shown that a comparatively short ischemic period of 30 minutes, from which the normal rat almost completely recovers, causes a rapidly progressive nephropathy and end-stage renal failure in the diabetic rat.

Several investigators have addressed the question of kidney injury in rodent diabetic models. Evan et al found only mild vacuolization in tubular cells after eight weeks of diabetes mellitus (DM), which was ameliorated by insulin treatment [6]. In streptozotocin (STZ)-DM kidneys followed for 30 weeks, Yong and Bleasel observed sparse fibrinoid glomerular lesions [4]. Hirose et al studied STZ-DM for up to 18 months, but still advanced lesions did not develop [2]. This is in accordance with the finding in the present study that lesions were sparse in the non-ischemic DM kidneys. These kidneys exhibited hypertrophy and hyperfiltration typical for DM rats [4, 16]. The hyperfiltration is known to be further increased by unilateral nephrectomy [3]. From a functional point of view, the unilateral ischemia in the DM animals in the present study may be considered equivalent to unilateral nepherctomy. This would explain the hypertrophy and hyperfiltration observed in the contralateral kidney.

The renal damage observed in the DM animals after ischemia differs substantially from that seen after ischemia-reperfusion in non-DM animals. The present injury is clearly progressive, whereas renal ischemia in the non-DM rat causes a functional impairment that is most pronounced one to two weeks after the ischemia and is followed by substantial recovery [17, 18]. In the present study 30 minutes of ischemia resulted in an almost completely reversible injury in the non-DM rats. With more extensive ischemia, that is, 60 minutes or more, the outcome may be a substantial loss of kidney mass and renal function even in non-DM animals. Nevertheless, in such animals maximal recovery is completed within four to eight weeks [18].

In principal the present injury could be explained in two ways. One possibility is an extreme susceptibility of the diabetic kidney to ischemia and the other is the presence of some yet unknown mechanism in diabetes that is triggered by ischemia and subsequently drives an inflammatory and fibrotic process.

Increased sensitivity to ischemia may be expected from the increase in Na,K-ATPase activity and thereby the increased oxygen demand along most segments of the nephron in STZ-diabetic rats [19]. Furthermore, hyperglycemia is associated with an increased ratio of free NADH/NAD+ similar to that found in hypoxia. This so-called pseudohypoxic state is associated with abnormalities in several cellular pathways [20]. Ischemia is likely to increase the NADH/NAD+ ratio further.

In the diabetic rat an increased sensitivity to renal ischemia has been demonstrated previously. Wald et al found that diabetic rats subjected to one hour of bilateral renal artery clamping died within 48 hours [11], while most non-diabetic animals survived the ischemic injury. A delayed recovery of oxygen consumption in slices from diabetic rat kidneys subjected to ischemia has been reported [13, 14], and it has been suggested that a more pronounced decrease in renal function after ischemia in diabetic rats may be due to an impaired response to nitric oxide in these animals [15].

All these previous studies, however, have concerned the early phase of ischemic injury. Further, they have used a more severe ischemic injury resulting from 60 minutes of clamping of the renal artery. This substantial ischemia will in most instances cause considerable irreversible kidney damage even in the normal, non-DM rat [18]. In the present study the ischemic period was only 30 minutes and in the non-DM rats was associated with almost complete recovery of renal function and morphology. In the diabetic rats, however, end-stage renal failure developed after eight weeks. Since the injury continues to progress from the fourth to the eighth week post-ischemia, the final injury is probably not entirely due to increased sensitivity to ischemia *per se*. In addition, ischemia-reperfusion seems to trigger some other, yet unidentified mechanism that comes into play and drives an inflammatory-fibrotic process affecting primarily the tubules and interstitium. In the present model glomerular injury seems to be a subsequent event. Glomerular atrophy and glomerulosclerosis were not pronounced until eight weeks after ischemia.

The extensive tubulointerstitial inflammation in the present study may at first seem contradictory to the well established view that glomerular injury is the hallmark of diabetic nephropathy. More than 30 years ago, however, Thomsen [7] observed in a series of renal biopsy specimens from patients with diabetic nephropathy that round cell infiltration was evident in a majority of the patients, indicating chronic pyelonephritis, although no correlation to bacteriuria or other signs of urinary infection was

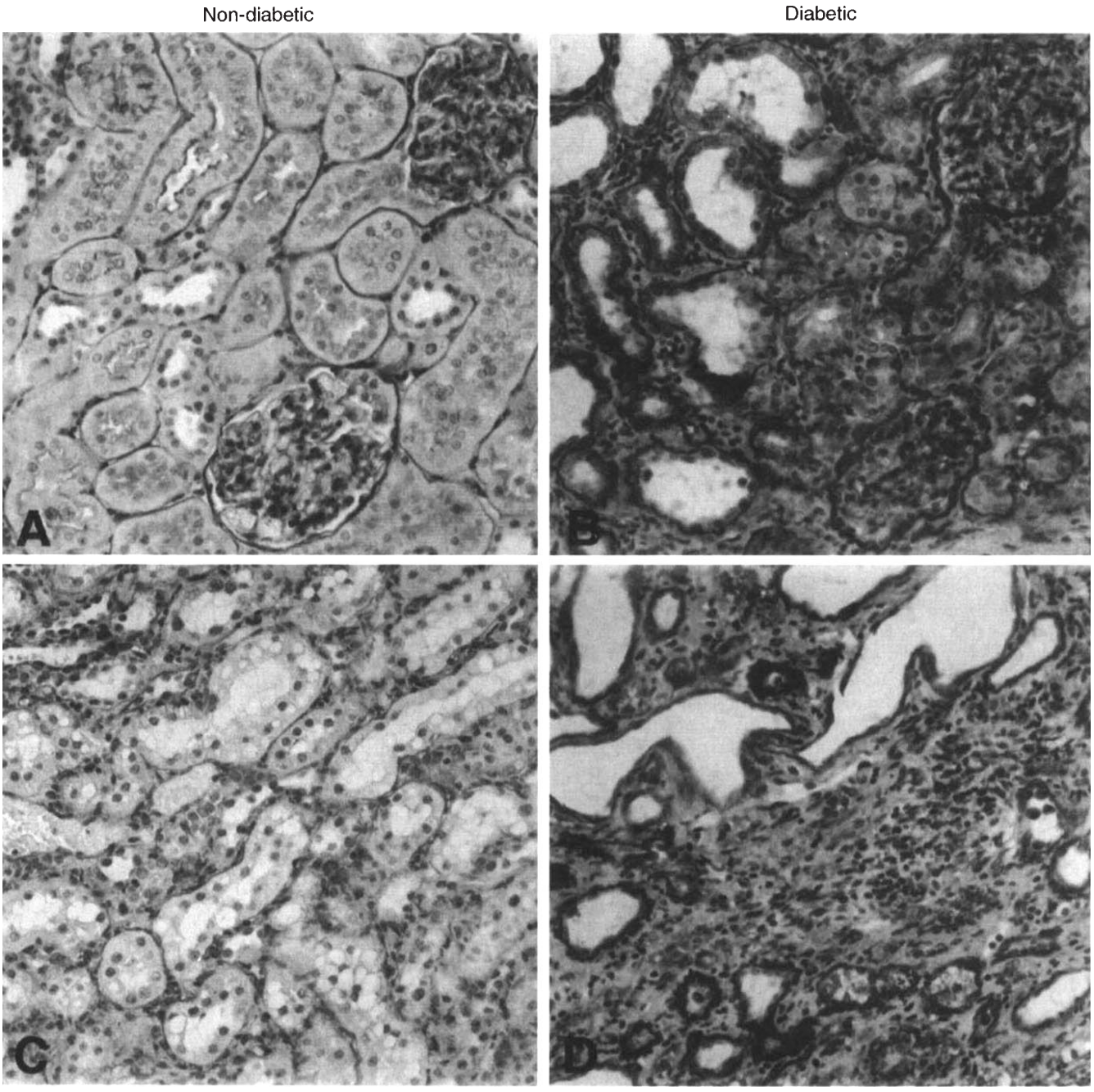

Fig. 3. Photomicrographs from kidneys eight weeks post-ischemia, PAS-staining. Cortical section from non-DM rat (*A*). Cortical section from DM rat (*B*). Tubular dilation and infiltration of inflammatory cells could be noted. Medullary section from non-DM rat (*C*). Medullary section from DM rat. Tubular dilation and atrophy is present along with pronounced inflammation (*D*). Magnification ×495.

found. He noted that the round cell infiltrate increased with the duration of diabetes and the degree of vascular obliteration. Thomsen suggested, in fact, that ischemia might be responsible for these changes in the diabetic patient. Other pathologists have pointed out that inflammation in renal biopsy specimens from patients with diabetes mellitus sometimes can be as extensive as to suggest an interstitial nephritis [10]. It has been proposed that the renal fibrosis that is a constant finding in diabetic nephropathy may be due to ischemia [10] and/or inflammation [9]. Thus, tubulointerstitial inflammation and fibrosis are also prominent features in human diabetic nephropathy. Furthermore, the interstitial lesions correlate with renal function in humans at least as well as do the glomerular lesions [21, 22].

Dehydration, which may aggravate ischemia-reperfuson injury, could be suspected in our study on the basis of the very extensive osmotic diuresis resulting from hyperglycemia. However, the high urine flow rate and GFR and the lower hematocrit values found in DM, as compared to non-DM animals, contradict the idea of

volume deficiency. Although the average values for arterial blood pressure were very low in the present study, most likely because of the anesthetic drug used, there was no difference in this respect between DM and non-DM animal. The low arterial blood pressures may, however, explain the low values for GFR as compared to those in rats anesthetized with barbiturates, for example.

The poor metabolic control in the STZ-DM rats may obviously play a role in the pathogenesis of the observed lesions. However, it should be emphasized that the DM animals in the present model are not completely without insulin production. For instance, neither ketonuria nor metabolic acidosis occurred. Other investigators have used larger doses of STZ, which result in complete inability to produce insulin, in which case insulin replacement is required to avoid ketoacidosis.

Tubular dilation was extensive in DM animals after ischemia. In contrast to the findings in a pure ischemic injury [17], intratubular casts were not a prominent finding. The present tubular dilation is most likely a consequence of inflammation, fibrosis and tubular atrophy of the tubular segment distal to the dilated tubule. The osmotic diuresis may also contribute, since tubular dilation was more pronounced in the non-ischemic DM kidneys as compared with non-DM animals, although this difference was not significant.

In the present model the destruction of the kidney seems to start with tubular-interstitial inflammation with fibrosis, while glomerular lesions and renal atrophy are subsequent features. When comparing the present model with human diabetic nephropathy, however, one must keep in mind that the latter takes several decades to evolve. In the present experimental model end-stage renal failure is attained within eight weeks. It follows that any mechanisms potentially involved in the development of diabetic end-stage renal failure, for instance tubulointerstitial inflammation and fibrosis, will be greatly amplified in the present rat model. One must also consider the differences in metabolic control and the type of ischemic injury. Obviously, complete ischemia and reperfusion does not occur in humans. Nevertheless, renal hypoxia and reperfusion oxidant stress may result from various physiological and pathophysilogical and conditions. Regional renal hypoxia from arterial obliteration most certainly occur in diabetic nephropathy.

In conclusion, the present study shows that ischemia causes rapidly progressive kidney damage in diabetic rats characterized by tubulointerstitial inflammation and fibrosis, while glomerular atrophy seems to be a subsequent feature. As severe lesions never develop in rodents with diabetes mellitus, the study suggests an experimental model that may easily be applied for further studies on the pathophysiology of progressive renal failure in diabetes.

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