Intervention with the aldose reductase inhibitor, tolrestat, in renal and retinal lesions of streptozotocin-diabetic rats

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Summary. The progressive increase in urinary albumin excretion, which precedes the development of diabetic nephropathy, can be prevented in diabetic rats if the aldose reductase inhibitor, tolrestat, is administered at the initiation and throughout the duration of hyperglycaemia. We therefore determined the ability of tolrestat to intervene in the further progression of already established urinary albumin excretion of streptozotocin-diabetic female Wistar rats. Two months after streptozotocin injection, diabetic rats were grouped as low-urinary albumin excretion (0.2–1.0 mg albumin/day) or high-urinary albumin excretion (1.9–5.9 mg albumin/day), at which time tolrestat intervention (25 mg/kg per day) was begun for half of the diabetic rats in each urinary albumin excretion group. After six months of treatment tolrestat caused

a significant reduction in the urinary albumin excretion rate of the low-urinary albumin excretion group only. The diabetes-induced rise of total urinary protein in both groups was significantly reduced by tolrestat. Furthermore, the diabetes-induced increase (49%) in the thickness of the basement membranes of retinal capillaries from the outer plexiform layer was significantly diminished by tolrestat administration. In conclusion, intervention therapy with the aldose reductase inhibitor, tolrestat, can reduce the progression of urinary albumin excretion and retinal basement membrane thickening in long-term diabetic rats.

Key words: Nephropathy, retinopathy, diabetic complications, aldose reductase.

The long-term hyperglycaemia associated with both Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes mellitus results in the slow development of multiple secondary complications. Diabetic nephropathy, one of the three major long-term complications, occurs in 30–40% of the diabetic population [1]. The early stages of diabetic nephropathy are characterized by an elevation of urinary albumin excretion (UAE) [2]. The albuminuria progresses to overt proteinuria, finally resulting in end-stage renal failure in diabetic patients. In confirmation of previous studies [3, 4], we found that six months of streptozotocin-induced diabetes in rats caused a substantial increase in the UAE. Furthermore, treatment of the diabetic rats with the aldose reductase inhibitor, tolrestat, for the entire duration of the 6-month experiment prevented the development of albuminuria [5].

A second major long-term complication of diabetes, retinopathy, is one of the leading causes of blindness [6]. Thickening of basement membranes occurs in the vessels of several tissues of diabetic patients and has been considered to be a hallmark of diabetes [7–9]. The capillary basement membranes of the retina normally increase in thickness with age [10]; this process is accelerated by diabetes [7,

8, 11]. Treatment of galactosaemic or BB diabetic rats with aldose reductase inhibitors prevents the diabetes-like thickening of capillary basement membranes [12–14].

The above studies suggest the potential usefulness of aldose reductase inhibitors in preventing the progression of diabetic nephropathy and retinopathy. Previously it has been demonstrated that vigilant control of hyperglycaemia by insulin treatment in diabetic patients and rats can reverse the progressive elevation of established albuminuria [15–17]. For this reason it was important to assess the effects of intervention therapy with the aldose reductase inhibitor, tolrestat, on albuminuria and retinal capillary basement membrane thickening in streptozotocin-diabetic rats.

Materials and methods

Animal treatments

Female Wistar rats (Charles River Breeding Laboratories; 200 g and 10 weeks of age at the beginning of the study) were used. Streptozotocin (STZ) (45 mg/kg) was injected into the tail vein of 369 rats and

a vehicle (0.03 mmol/l citrate, pH 4.5) was administered to 22 rats. Two months after the administration of STZ, 122 of the 306 surviving diabetic rats were separated into two sets, based on their urinary albumin levels (low UAE, $n = 46$; high UAE, $n = 76$). The diabetic rats having low (< 0.9 mg/day) (Groups 2 and 3) or high (2.0–6.0 mg/day) (Groups 4 and 5) levels of UAE were then randomly assigned to a drug (tolrestat)- or non-drug-treated group. The remaining 184 diabetic rats were eliminated from the study for one or more of the following reasons: 1) the UAE level at month 2 was either between 0.9 to 2.0 mg/day or > 6.0 mg/day; 2) the plasma glucose level of an STZ-injected rat was either < 16.8 or > 44.8 mmol/l; 3) the urine volume of an STZ-injected rat was < 100 ml/day, or there was a substantial (> 20 g) loss of body weight during month 2, and therefore the general health of the animal was probably not adequate to survive the remaining 6 months of the experiment.

At monthly intervals (months 0 and 1–8), the rats were placed individually into metabolism cages (Nalgene Co.; Rochester, N. Y., USA) (ad libitum food and 150 ml water) for 24 h collection of urine. Total volume of urine was recorded, aliquots of urine were centrifuged ($100 \times g$, 10 min) to remove particulate matter, and multiple aliquots were frozen at -70°C . The concentration of tolrestat admixed to the ground chow (3.5–4.0 g tolrestat per 25 kg chow) was adjusted weekly to provide an average daily dose of 25 mg/kg, based on the mean body weight and food consumption of the drug-treated rats. This dose of tolrestat was used since it had been shown previously in rats to prevent diabetes-induced albuminuria and renal accumulation of sorbitol [5]. Since we have demonstrated previously that tolrestat treatment (6 months) does not alter UAE levels in non-diabetic rats [5], a tolrestat-treated non-diabetic control group was not included. All animals were given free access to water and food and were maintained under a 12 h on/off light cycle with cage-level illuminations of 15 to 30 foot candles. Animal care and treatment conformed to the NIH Guide for the Care and Use of Laboratory Animals. After 8 months of diabetes, the surviving rats were anaesthetized, kidneys perfused with 154 mmol/l NaCl, renal cortex and inner medulla were separated and rapidly frozen.

Electron microscopy

Eyes from a random sample of rats from each group were fixed by immersion for 24 h or more at room temperature in a solution of 2.5% glutaraldehyde and 6% sucrose, buffered to pH 7.2 with 50 mmol/l sodium cacodylate. Small portions (< 1.0 mm²) of retina were taken from within 1.5 mm of the optic disk of the superior temporal quadrant. The specimens were post-fixed in OsO₄ buffered with 150 mmol/l Na-K phosphate (pH 7.4), embedded, sectioned, stained and examined using a JEM 100B electron microscope (JEOL USA Inc.; Peabody, Mass., USA). Micrographs of capillaries of the outer plexiform layer were taken at a magnification of $\times 5,000$. Only those capillaries which were transected in a perpendicular or near-perpendicular direction and showed unambiguous basement

membrane borders were selected for analysis. Quantification of basement membrane thickness was performed using a Bioquant II digitizing morphometric system (R & M Biometrics; Nashville, Tenn., USA) [18]. The mean areas for all basement membranes of the retinal capillaries, including those enveloping the pericytes, were determined and the mean values were divided by the total length of the basement membranes [12]. The measurements were expressed as mean basement membrane thickness in nanometers. The procedures were carried out under masked conditions with 20 micrographs of capillaries from each of either six age-matched non-diabetic control rats or 12 diabetic rats randomly selected from each experimental group.

Biochemical methods

Glucose and sorbitol in tissue samples were converted to their aldonitrile acetate derivatives [19] and quantitated by capillary gas chromatography. Using a VP Analyzer (Abbott Laboratories, North Chicago, Ill., USA) aliquots of plasma were assayed for glucose, creatinine, urea nitrogen, and triglycerides, and urine samples were assayed for glucose and creatinine. Total urinary protein was determined by a microtitre plate assay using the BCA Protein Assay Reagents (Pierce, Rockford, Ill., USA). Levels of albumin in the urine from individual rats were determined by an ELISA used previously [5].

Statistical analysis

Because of the unequal variance among groups, raw data were usually log transformed prior to statistical analysis and the baseline UAE rate (i.e., mean of months 1 and 2) was subtracted from the UAE rate of months 3–8 to adjust for differences in baseline UAE. Since animal selection into treatment group at month 2 was dependent upon baseline UAE levels, statistical evaluation of drug effects was performed separately for rats of the low-UAE (Groups 2 and 3) and high-UAE (Groups 4 and 5) groups. The transformed data were analysed using either a repeated measures analysis of variance or a one-way analysis of variance. The least significant difference between groups was considered statistically significant if $p < 0.05$.

Results

At the termination of the experiment (month 8) all of the surviving STZ-injected rats demonstrated significant polyuria, glycosuria and a lower body weight than non-diabetic rats (Table 1). Fed plasma levels of glucose and triglycerides were substantially elevated, whereas plasma

Table 1. Characteristics of animals in each group at end of experiment (month 8)

No.	Group Condition	<i>n</i>	Body weight (g)	Urine			Plasma			
				Volume (ml/day)	Glucose (mmol/day)	Creatinine ($\mu\text{mol/day}$)	Glucose (mmol/l)	Triglycerides (mmol/l)	Creatinine ($\mu\text{mol/l}$)	BUN (mmol/l)
1	Non-diabetic	22	324 $\pm$ 7 ^a	27 $\pm$ 2	(11 $\pm$ 6) ^b	136 $\pm$ 4	8.3 $\pm$ 0.2	1.23 $\pm$ 0.1	27 $\pm$ 9	8.2 $\pm$ 0.4
2	Diabetic									
2	Low UAE	21	258 $\pm$ 6 ^d	125 $\pm$ 7 ^d	49 $\pm$ 2 ^d	200 $\pm$ 12 ^d	33.2 $\pm$ 0.8 ^d	8.20 $\pm$ 1.36 ^d	88 $\pm$ 9 ^d	12.5 $\pm$ 0.4 ^d
3	Low UAE + tolrestat ^c	18	263 $\pm$ 9 ^d	127 $\pm$ 3 ^d	49 $\pm$ 2 ^d	182 $\pm$ 7 ^d	33.0 $\pm$ 1.3 ^d	7.66 $\pm$ 1.91 ^d	71 $\pm$ 9 ^d	12.5 $\pm$ 0.7 ^d
4	High UAE	30	235 $\pm$ 8 ^d	137 $\pm$ 4 ^d	46 $\pm$ 2 ^d	191 $\pm$ 10 ^d	36.2 $\pm$ 0.8 ^d	16.02 $\pm$ 2.59 ^d	88 $\pm$ 9 ^d	15.0 $\pm$ 0.7 ^d
5	High UAE + tolrestat ^c	32	236 $\pm$ 7 ^d	137 $\pm$ 4 ^d	48 $\pm$ 2 ^d	182 $\pm$ 7 ^d	35.3 $\pm$ 1.0 ^d	13.99 $\pm$ 1.51 ^d	97 $\pm$ 9 ^d	15.4 $\pm$ 0.7 ^d

^a Values are mean $\pm$ SEM; ^b Value of Group 1 is reported as $\mu\text{mol/day}$; ^c Daily dose of 25 mg/kg admixed to the diet; ^d $p < 0.05$ vs Group 1; ^e $p < 0.05$ vs respective non-treated diabetic group.
UAE = urinary albumin excretion; BUN = blood urea nitrogen

Table 2. Carbohydrate levels in kidney at end of experiment (month 8)

No.	Group		Renal cortex		Inner renal medulla	
	Condition	<i>n</i>	Glucose	Sorbitol	Glucose	Sorbitol
1	Non-diabetic	22	0.5 ± 0.1 ^a	0.02 ± 0.00	0.3 ± 0.0	0.98 ± 0.10
2	Diabetic					
2	Low UAE	21	5.1 ± 0.4 ^b	0.10 ± 0.01 ^b	7.5 ± 0.6 ^b	1.55 ± 0.21 ^b
3	Low UAE + tolrestat	18	5.9 ± 0.6 ^b	0.05 ± 0.01 ^{b,c}	8.2 ± 0.7 ^b	0.30 ± 0.04 ^{b,c}
4	High UAE	30	7.9 ± 0.6 ^b	0.13 ± 0.01 ^b	7.3 ± 0.5 ^b	0.71 ± 0.06 ^b
5	High UAE + tolrestat	32	7.2 ± 0.5 ^b	0.05 ± 0.01 ^{b,c}	8.8 ± 0.7 ^b	0.15 ± 0.01 ^{b,c}

^a Values are nmol/mg tissue, mean ± SEM; ^b $p < 0.05$ vs Group 1; ^c $p < 0.05$ vs respective non-treated diabetic group. UAE = urinary albumin excretion

Table 3. Urinary albumin excretion (UAE) at month 2 (pre-treatment)

No.	Group		UAE (mg/day)	
	Condition	<i>n</i>	Mean ± SEM	range
1	Non-diabetic	22	0.5 ± 0.1	0.1–3.4
2	Diabetic			
2	Low UAE	23	0.6 ± 0.1	0.3–1.0
3	Low UAE + Tolrestat ^a	23	0.6 ± 0.1	0.2–1.0
4	High UAE	38	3.5 ± 0.2 ^b	2.1–5.9
5	High UAE + Tolrestat ^a	38	3.6 ± 0.2 ^b	1.9–5.9

^a Tolrestat was administered during months 3–8 only; ^b $p < 0.05$ vs Group 1

levels of creatinine and urea nitrogen were only slightly increased. None of the above parameters were significantly altered by tolrestat treatment. Similar plasma glucose levels were also obtained after 2 and 4 months of diabetes. In contrast, however, administration of tolrestat to the diabetic rats significantly reduced the tissue levels of sorbitol at month 8 (Table 2).

Urinary protein

Two months after the injection of STZ, the 306 diabetic rats had UAE levels ranging from 0.1 to 32.4 mg/day. Of these diabetic rats 122 were distributed (see Methods) into Groups 2–5 such that the UAE levels of Groups 4 and 5 (high UAE), but not Groups 2 and 3 (low UAE), were

significantly different from the UAE level of the non-diabetic rats at month 2 (Table 3).

The UAE level of all non-diabetic and diabetic rats increased throughout the 8-month experimental period (Fig. 1). During months 3–8, the diabetic rats in the high UAE groups continued to have substantially greater UAE levels than those of the low UAE groups. After 6 months of treatment, reductions of UAE by tolrestat were observed in both groups [low UAE (–53%) and high UAE (–36%)] which, when adjusted for baseline levels (mean level of months 1 and 2), were significantly lower in the low UAE group only.

Unlike urinary albumin excretion, the daily excretion of total protein increased abruptly with the onset of diabetes (Fig. 2), paralleling the diabetes-induced changes in urine volume (data not shown). After 4 to 6 months of diabetes, the levels of urinary total protein of all groups began to increase gradually. The reduction in total protein excretion by tolrestat was significant at month 8 for the low UAE group and months 7 and 8 for the high UAE group.

After 8 months of hyperglycaemia, the UAE levels of non-treated diabetic rats were highly correlated with the plasma triglycerides ($r = 0.70$, $p < 0.001$). There was a similar, but less pronounced, correlation of UAE and plasma triglyceride levels at month 8 in the diabetic rats treated with tolrestat ($r = 0.45$, $p < 0.01$). In contrast, the plasma glucose levels of the diabetic rats were not significantly correlated with the rate of UAE ($r = 0.31$ and $r = 0.22$, $p > 0.05$ for both non-treated and treated diabetic rats, respectively).

Fig. 1. Effect of intervening tolrestat treatment (months 3–8) on urinary albumin excretion (UAE) of diabetic rats in either the low-UAE (left) or high-UAE (right) groups. * $p < 0.05$ vs diabetic group using log values adjusted for baseline levels. Non-diabetic (■); diabetic (▨); diabetic + tolrestat (□)

Fig. 2. Effect of intervening tolrestat treatment (months 3–8) on urinary total protein of diabetic rats in either the low-UAE (left) or high-UAE (right) groups. * $p < 0.05$ vs diabetic group using log values. Non-diabetic (■); diabetic (▨); diabetic + tolrestat (□)

Retinal basement membrane

The eyes of a randomly chosen sample of rats from each experimental group ($n = 6$ per group) were used to assess changes in retinal capillary basement membrane thickness. Since there was an insignificant difference in basement membrane thickness between the rats of the low and high UAE groups the data of the rats in both groups were pooled. Eight months of hyperglycaemia induced a substantial (49%) increase over that of age-matched controls in the thickness of the basement membranes of retinal capillaries from the outer plexiform layer (Fig. 3). Intervening treatment of the diabetic rats with tolrestat for the last 6 months of diabetes, significantly inhibited the basement membrane thickening.

Not only were the basement membranes thicker (Fig. 4) in the diabetic as compared to the non-diabetic rats, but they also contained more fibrous collagen of the banded type and more clear vacuoles and dense inclusions. The basement membranes in the retinal capillaries of the outer plexiform layer appeared normal, in both

average thickness and in structural content, in the tolrestat-treated rats.

Discussion

Our previous study [5] demonstrated that diabetes-induced elevations of UAE could essentially be prevented by administration of tolrestat to rats during the entire period of hyperglycaemia. Using a variety of aldose reductase inhibitors (e.g., tolrestat, sorbinil and statil), many other researchers have confirmed this finding [20–22]. It should be noted, however, that there have been a few reports in which, for reasons that are not apparent, conflicting results have been obtained [23, 24]. There is substantial evidence that the elevation of UAE in diabetic rats is due, at least in part, to the increased flux of glucose through the polyol pathway [25].

In clinical trials, the efficacy of an aldose reductase inhibitor will be assessed initially only after patients exhibit a symptom of diabetic nephropathy, such as albuminuria. Vigilant control of hyperglycaemia in diabetic patients and rats can further reduce increases in existing albuminuria [15–17]. To better approximate clinical trials, it was essential to evaluate the ability of an aldose reductase inhibitor, such as tolrestat, to retard further progression of established albuminuria in diabetic rats.

The ability of tolrestat to slow the progressive worsening of albuminuria was evident in the low UAE group after approximately 3 months of drug treatment and was statistically significant after 6 months of treatment (i.e., at month 8). Within the limited period that the diabetic rats were treated and evaluated, there was no evidence that tolrestat reversed the albuminuria, only that it retarded further progression. A major observation of this study is the dramatic difference in the apparent efficacy of tolrestat when administered in prevention rather than intervention treatment regimens (i.e., comparison of previous data [5] to present data). Although albuminuria has been suggested to be one of the initial symptoms of diabetic nephropathy, substantial and irreversible renal damage may already exist at this early stage [26, 27]. If irreversible pathologies are present, then drug therapy could be completely successful only if it was initiated prior to the advent

Fig. 3. Effect of intervening tolrestat treatment (months 3–8) on retinal capillary basement membrane thickness in 8-month diabetic rats. * $p < 0.05$ vs diabetic group. $n = 6$, 12 and 12 for non-diabetic (■); diabetic (▨); diabetic + tolrestat (□) groups, respectively

Fig. 4A–C. Representative transections of retinal capillaries from the outer plexiform layer (deep capillary bed) of non-diabetic (A); diabetic (B); and diabetic + tolrestat (C) rats showing the basement membrane (BM and ()); pericytes (P), endothelial cells (E), and lumen (L) of each (x 14,000)

of albuminuria, or at least as early as possible, and only if the treatment period was sufficiently long to allow significant repair of disease damage. Consistent with this premise, tolrestat had greater efficacy in the group of diabetic rats having the lowest UAE. However, additional factors are clearly involved since the urinary excretion of total protein by rats of both the high and low UAE groups was reduced by tolrestat treatment. Additional experiments, in which treatment is initiated at various times, are needed to assess whether the efficacy of tolrestat is more dependent upon the severity of albuminuria or the duration of hyperglycaemia.

Some non-diabetic rats exhibit high UAE rates, which can be comparable to the levels of diabetic rats [5, 28]. This observation is consistent with the premise that albuminuria in rats is not due entirely to hyperglycaemia. In fact, we previously found that the UAE rate in Zucker (fa/fa) rats was dependent upon age and obesity and independent of blood glucose levels [29]. Other researchers have demonstrated that the albuminuria, which occurs in obese Zucker rats, can be reduced by treatment with lipid-lowering agents [30]. Consistent with these reports, we observed that the UAE rates of diabetic rats were significantly correlated with their plasma triglyceride levels after 8 months of diabetes. Whether or not the treatment of either diabetic rats or patients with an antihyperlipidaemic agent is able to reduce the progression of albuminuria is not yet known. Interestingly, a correlation between the incidence rate of albuminuria and hypertriglyceridaemia of diabetic patients has recently been observed [31]. Furthermore, an increase in lipid levels in intestinal microvasculature and lymphatics during lipid absorption was associated with enhanced microvascular permeability to proteins [32]. Determination of the significance of these relationships awaits further experimentation.

An increase in the activation of aldose reductase has also been implicated in the basement membrane thickening, which occurs in the galactosaemic rat model of diabetic complications [12, 13]. In diabetic rats, retinal capillary basement membrane thickening has been well documented after 12 months diabetes-duration [33], and has been reported to occur as early as 2–4 months after onset [34–36]. However, only a few studies have provided data suggesting a possible beneficial effect of aldose reductase inhibitors [14, 35–37]. The present study further documents a significant thickening of the capillary basement membranes in the retinal capillaries of the outer plexiform layer (deep capillary bed) and demonstrates that intervention with tolrestat after 2 months of established diabetes inhibited the thickening. Studies involving additional sampling times are needed to determine if this inhibition of thickening merely represents halting at an early stage of thickening or a reversal of the thickening process. Interestingly, in a recent publication Chakrabarti and Sima [14] found a different aldose reductase inhibitor, statil, to also affect the basement membrane thickening of retinal capillaries of the outer plexiform layer, but not those of the superficial capillary bed. While in this study we have not investigated the possible effects of tolrestat on the retinal basement membranes of the superficial ca-

pillary bed, in previous experiments tolrestat was effective in both capillary beds of galactose-fed rats [38].

In conclusion, intervention therapy of STZ-diabetic rats with the aldose reductase inhibitor, tolrestat, significantly inhibited the thickening of retinal capillary basement membranes and the progression of albuminuria in the diabetic rats having initially low rates of UAE. The findings from this study, and others [18, 39–41], suggest that early treatment of diabetic patients with aldose reductase inhibitors may delay or possibly prevent fundamental complications associated with diabetic neuropathy, retinopathy, and nephropathy.

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