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Prevention of Diabetes-Induced Albuminuria in Transgenic Rats Overexpressing Human Aldose Reductase

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Studies using pharmacologic inhibitors have implicated the enzyme aldose reductase in the pathogenesis of albuminuria and diabetic renal disease. However, a clear conclusion is not easily drawn from such studies since these pharmacologic inhibitors have nonspecific properties. To examine further the role of aldose reductase, we have overexpressed the human enzyme in a transgenic rat model. Transgene expression in the kidney was predominantly localized to the outer stripe of the outer medulla, compatible with the histotopography of the straight (S3) proximal tubule. The effect of enzyme overexpression on diabetes-induced renal function and structure was then investigated. Contrary to what may have been anticipated from the previous enzyme inhibition studies, diabetes-induced albuminuria was completely prevented by the overexpression of aldose reductase. No effect of overexpression of aldose reductase on renal structure nor on urinary excretion of β_2-microglobulin and *N*-acetyl-β-D-glucosaminidase was observed in this transgenic rat model. In conclusion, our study strongly suggests that multiple roles for aldose reductase may give it a more complex place in diabetic nephropathy than is currently recognized.

Key Words: Hyperglycemia; diabetic nephropathy; cytomegalovirus; proximal tubule; carbonyl stress.

Introduction

Aldose reductase (AR_2, EC 1.1.1.21) is a monomeric NADPH-dependent cytosolic enzyme that catalyzes the reduction of a broad range of carbonyl-containing compounds to their respective alcohols. As the key enzyme of the polyol pathway, aldose reductase converts glucose to sorbitol with the concomitant oxidation of NADPH to $NADP^+$. In tissues in which sorbitol dehydrogenase is expressed together with aldose reductase, sorbitol may then be oxidized to fructose.

In diabetes, metabolic imbalances resulting from excessive polyol pathway flux are thought to culminate in adverse changes in renal structure and function. Evidence for this conclusion comes primarily from studies in diabetic rodents treated with pharmacologic aldose reductase inhibitors (ARIs). Although beneficial effects of ARIs have not been observed in all studies, these inhibitors have been found to exert a positive influence on functional and structural aspects of the kidney including urinary albumin and protein excretion in the relative short term *(1–6)*, glomerular filtration rate (GFR) *(6–9)*, mesangial expansion *(10,11)*, glomerular basement thickening *(6,10)*, and glomerular hypertrophy *(6)*. However, these studies should be interpreted with caution since the consequences arising from any nonspecific effects of these chemicals in these experiments have not been directly assessed *(12–15)*.

Therefore, the role of aldose reductase in diabetic nephropathy must be further evaluated using different experimental approaches. To accomplish this task, we have developed a transgenic rat model specifically engineered to overexpress human aldose reductase. We report here the use of this animal model in investigating the significance of aldose reductase in the development of renal functional complications.

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Results

Generation and Characterization of Transgenic Rats Overexpressing Human Aldose Reductase

Both male and female transgenic PVG/c F_0 founders were produced by pronuclear microinjection. Transgenic status was indicated by the presence of a predicted 789-bp band (in addition to an ~3.3-kb band) in *Bam*HI-digested tail genomic DNA in Southern analysis using the entire transgene as probe (Fig. 1). A line of homozygous transgenic rats ($TgCMVhAR_2$) was established from one of these F_0 rats. Homozygosity in this line was confirmed using both

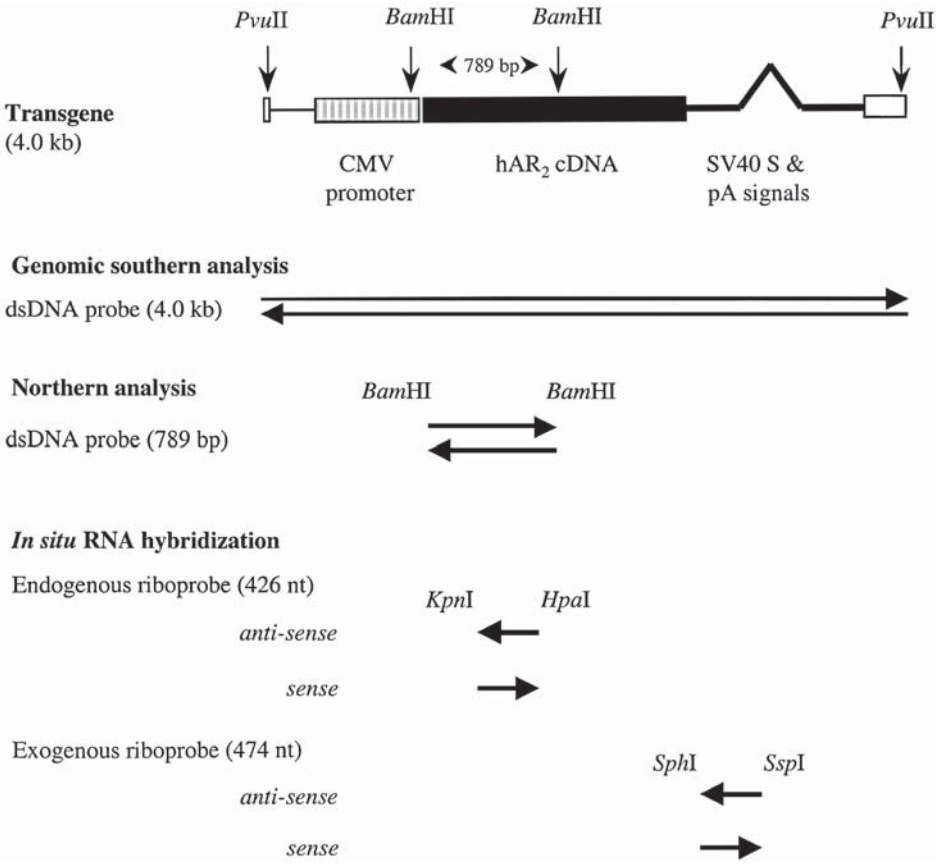

Fig. 1. Representation of transgene and nucleic acid probes used in production and molecular characterization of TgCMVhAR$_2$ transgenic rats overexpressing human aldose reductase. Unlabeled portions of the transgene refer to vector sequences derived from pCMVhAR$_2$ following *Pvu*II digestion.

genomic Southern analyses and conventional backcrossing over four generations. Additional transgenic lines were bred from three individual F_0 rats. A line of PVG/c control rats was also established from nontransgenic littermates of the F_0 rats.

Northern analysis was performed on total RNA from tissues obtained from TgCMVhAR$_2$ rats (Fig. 2). A 1.4-knt transcript representing endogenous rat aldose reductase mRNA was present in all tissues except the adult liver. An ~2.4-knt transcript originating from the cytomegalovirus (CMV) promoter-driven transgene was present in the retina, testes, aorta, ocular lens, and kidney (Fig. 2). Unlike the endogenous rat aldose reductase gene, transgene expression was not observed in isolated glomeruli (data not shown). *In situ* RNA hybridization detected endogenous aldose reductase mRNA strongly in the renal papillae of both transgenic and control rats (Fig. 3). By contrast, expression of the transgene in TgCMVhAR$_2$ kidneys was predominantly in the outer stripe of the outer medulla (Fig. 3); homogeneous labeling of the proximal tubules was compatible with the histotopography of the straight (S3) proximal tubule (data not shown). Little or no transgene expression was seen in the tubules or glomeruli of the renal cortex (Fig. 3).

Northern analysis was performed on total RNA extracted from heart, kidney, aorta, skeletal muscle, ocular lens, and retina of three other transgenic lines descended from separate F_0 rats. Exogenous aldose reductase mRNA was present in varying degrees in these tissues in two lines but was absent in the third. Endogenous aldose reductase mRNA was expressed in these tissues examined from these lines (data not shown).

To determine whether the transgene resulted in increased renal aldose reductase protein, Western immunoblot analysis using a polyclonal hAR$_2$ antibody was performed on homogenates prepared from TgCMVhAR$_2$ and control kidney sections from which renal papillae had been either removed by microdissection or left intact. Densitometric analysis of Western immunoblots indicated that TgCMVhAR$_2$ kidney samples (containing cortex and medulla but without papillae) had significantly greater amounts of aldose reductase protein compared with control kidneys ($n = 9$ in each group, $p < 0.05$) (Fig. 4). In homogenates prepared from whole

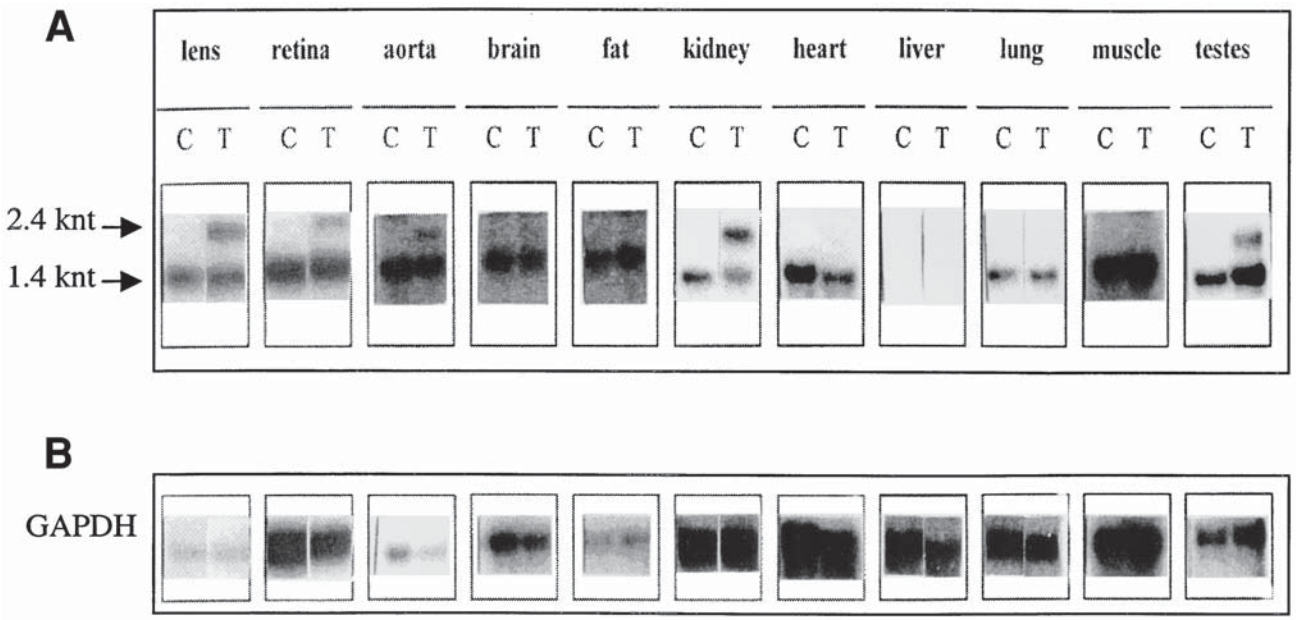

Fig. 2. Tissue expression of endogenous (1.4 knt) and transgene-derived aldose reductase (~2.4 knt) mRNAs in TgCMVhAR$_2$ rats. (**A**) Total RNA from tissues of transgenic (T) and wild-type rats (C) was probed with a 789-bp fragment of human aldose reductase cDNA. (**B**) rat GAPDH cDNA was used to normalize for RNA loading.

kidney sections with papillae intact, in which there was abundant rat aldose reductase protein, no significant differences were found in the level of this protein between transgenic and control rats whether nondiabetic or streptozotocin (STZ) diabetic (data not shown).

Effect of Aldose Reductase Overexpression on Diabetes Expression and Renal Parameters in Diabetes

The effect of overexpression of aldose reductase on renal function and structure in STZ-induced diabetic state was evaluated in a cohort of control and TgCMVhAR$_2$ rats over a period of 20 wk. This cohort consisted of four groups of six animals: diabetic and nondiabetic transgenic and diabetic and nondiabetic control rats. One diabetic transgenic rat was excluded from the evaluation when at sacrifice the macroscopic appearance of its kidneys revealed multiple cysts that were not observed in the rest of the transgenic and control animals. Inclusion of results from this animal would not have altered the findings significantly.

As a routine procedure, heavier age-matched rats were selected for induction of STZ diabetes. Diabetes was associated with less weight gain, polyuria, and polydipsia but there was no difference between transgenic and nontransgenic diabetic groups (Table 1). All diabetic rats recorded blood glucose levels >15 mmol/L throughout the experiment, and diabetic groups were significantly and equally hyperglycemic compared with their nondiabetic counterparts (Table 1). Glycated hemoglobin was significantly higher in nontransgenic and transgenic diabetic animals compared with their nondiabetic counterparts, with no significant difference between diabetic groups. Notably, there was a significant 25% reduction in glycated hemoglobin levels in nondiabetic transgenic rats compared with nondiabetic control rats (Table 1). This reduction occurred despite highly comparable blood glucose levels in these two groups throughout the entire study (Table 1).

Diabetes in both nontransgenic and transgenic animals was associated with kidney hypertrophy at the time of sacrifice. Significant renal enlargement was seen in both diabetic transgenic and control rats compared with their nondiabetic counterparts (Table 2). A twofold increase in kidney size as a result of diabetes was also observed when kidney index (percentage of kidney mass/body mass) was compared. No significant difference in kidney index was observed between both diabetic groups or both nondiabetic groups (Table 2). Morphometric study was performed on sections from perfused rat kidneys obtained at sacrifice. After 22 wk of diabetes, significant enlargements in the glomerular tuft as well as Bowman's area and in tubular cross-sectional area were seen in both control and transgenic animals (Table 2). The presence of the transgene did not alter these parameters in nondiabetic animals, nor modify the effect of diabetes.

The effect of transgene expression on albuminuria was assessed since previous studies in animal models had shown that ARIs could ameliorate this parameter. With the exception of nondiabetic transgenic animals, albuminuria increased

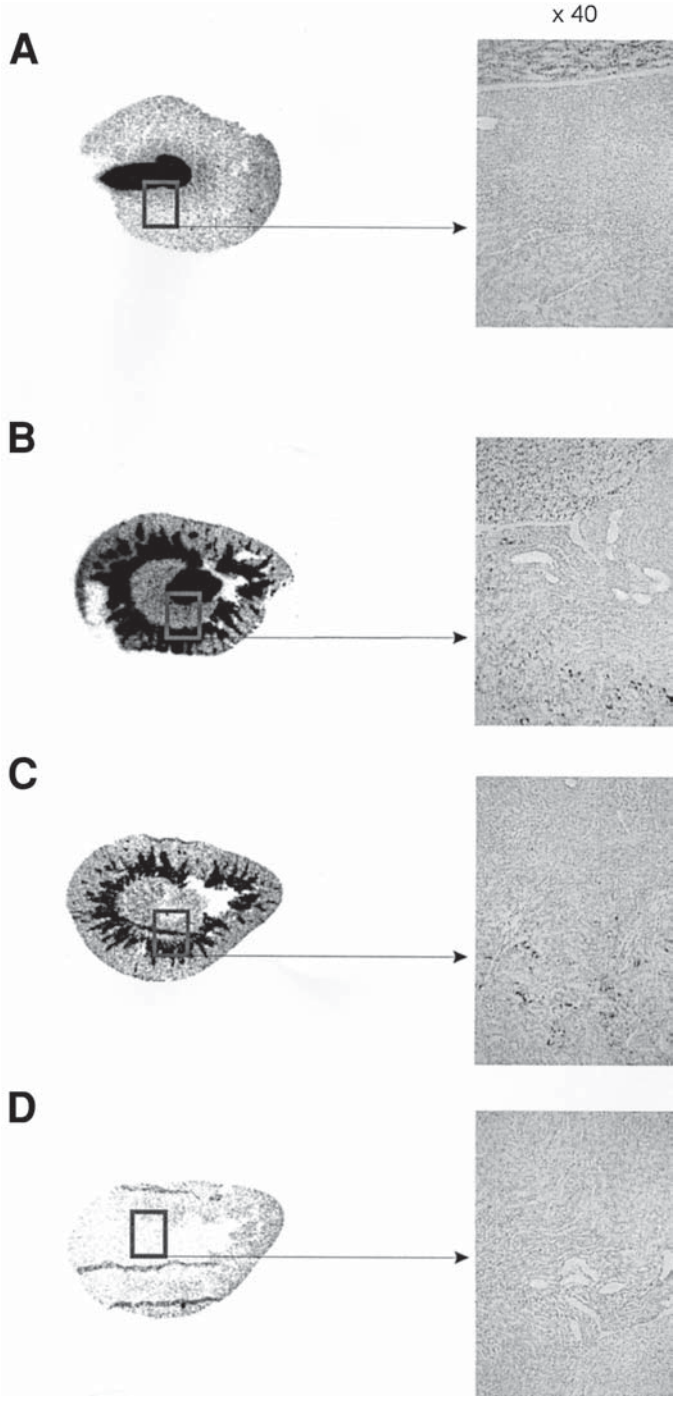

Fig. 3. In situ hybridization analysis of endogenous rat AR_2 and $TgCMVhAR_2$ transgene expression in nondiabetic PVG/c (control) and $TgCMVhAR_2$ kidney sections. An endogenous antisense riboprobe that detects both rat and transgene-derived human AR_2 mRNA was used to probe PVG/c control (**A**) and $TgCMVhAR_2$ kidney sections (**B**). Boxes indicate kidney sections shown at the right under low-power magnification. (**C**) $TgCMVhAR_2$ kidney section probed with exogenous antisense riboprobe specific for transgene-derived human AR_2 mRNA. (**D**) $TgCMVhAR_2$ kidney section showing absence of specific hybridization to sense endogenous riboprobe. Details of the riboprobes used are shown in Fig. 1.

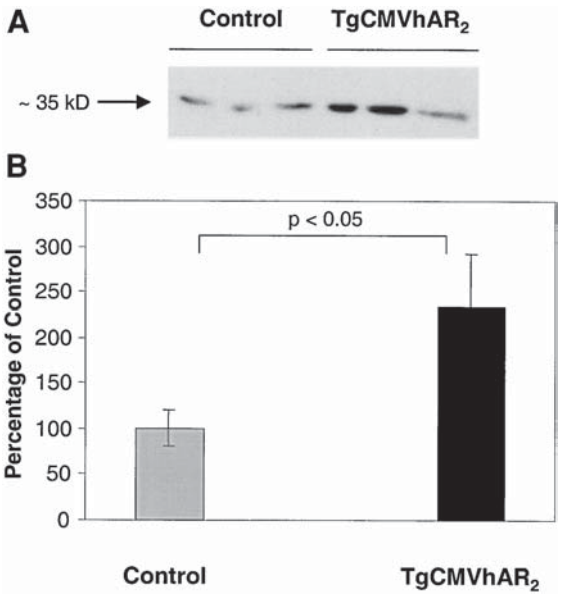

Fig. 4. Aldose reductase protein level in lysates of kidneys (containing cortex and medulla but without papillae) obtained from control PVG/c and transgenic $TgCMVhAR_2$ rats. (**A**) Representative Western immunoblot and (**B**) densitometric measurement of AR_2 protein levels ($n = 9$ for each group), expressed as percentage of control.

significantly with time in the three remaining groups (Table 2). This parameter was exacerbated by STZ diabetes in both diabetic groups but to a much greater degree in the diabetic nontransgenic group. At wk 20, albuminuria in diabetic control rats was significantly higher than in their nondiabetic counterparts. Although diabetic transgenic rats consistently excreted more albumin in their urine than respective nondiabetic counterparts, these differences were not statistically significant up to wk 20 (Table 2). Indeed, diabetic transgenic rats exhibited a very similar level of albumin excretion to that of nondiabetic control rats at this time point, indicating a near-complete amelioration of diabetes-induced albuminuria. At the earliest time point measured (9 wk), the level of albuminuria in diabetic control rats was asymptotically equal to twofold higher than that of transgenic counterparts although this was not statistically significant. At this time point, diabetic control rats had significantly higher albumin excretion compared with nondiabetic control rats, but, again, no significant difference was seen between the nondiabetic and STZ diabetic transgenic groups. In addition, nondiabetic transgenic rats excreted ~50% less albumin in their urine than nondiabetic controls at 20 wk. Thus, diabetes-induced and age-associated increments in albuminuria were decreased in $TgCMVhAR_2$ rats compared with their nontransgenic (control) counterparts.

Increased albumin excretion may be owing to elevations in the GFR. While GFR was not determined in the present study, no significant effect of transgene expression on creatinine clearance (a surrogate measure of GFR) was detected in separate cohorts ($n = 6$) examined after 3 wk (short dura-

Table 1
Effect of STZ Diabetes on General Body Parameters in Cohorts of Control and Homozygous TgCMVhAR$_2$ Transgenic Rats[a]

Body parameter	Time after diabetes onset (wk)	CON-ND (n = 6)	CON-D (n = 6)	TG-ND (n = 6)	TG-D (n = 5)
Body mass (g)	0	151 (7)[b]	194 (6)[b,g]	155 (4)[b]	200 (11)[b,i]
	2	244 (5)[b]	237 (11)[b]	248 (6)[b]	234 (7)[b]
	4	299 (4)[b]	262 (5)[b,g]	303 (7)[b]	261 (9)[b,i]
	9	371 (6)[b]	305 (11)[g]	374 (6)[b]	288 (10)[i]
	12	406 (6)[b]	327 (12)[g]	395 (7)[b]	299 (9)[i]
	16	430 (6)	334 (14)[g]	419 (7)	308 (10)[i]
	20	443 (7)	333 (15)[g]	435 (5)	304 (11)[i]
Water intake (mL/h)	9	1.20 (0.12)	5.32 (0.54)[g]	1.23 (0.04)	5.83 (0.72)[i]
	12	1.12 (0.09)[c]	5.43 (0.58)[g]	1.45 (0.04)[h]	6.00 (0.81)[i]
	16	1.20 (0.09)	5.87 (0.68)[g]	1.19 (0.09)	5.87 (0.76)[i]
	20	1.12 (0.07)	6.59 (0.66)[g]	1.39 (0.05)	6.39 (0.64)[i]
Urine production (mL/h)	9	0.43 (0.06)	4.50 (0.37)[g]	0.34 (0.02)	4.68 (0.68)[i]
	12	0.38 (0.02)[d]	4.88 (0.54)[g]	0.53 (0.04)[e]	5.15 (0.75)[i]
	16	0.40 (0.04)	4.89 (0.50)[g]	0.51 (0.04)[e]	4.94 (0.72)[i]
	20	0.51 (0.02)	5.73 (0.52)[g]	0.47 (0.03)	5.40 (0.65)[i]
Blood glucose (mmol/L)	9	5.8 (0.2)	21.9 (0.8)[g]	6.4 (0.2)	24.7 (1.4)[i]
	12	6.9 (0.1)[e]	26.4 (1.6)[g]	6.4 (0.2)	26.4 (1.5)[i]
	16	6.3 (0.2)	22.7 (1.6)[g]	6.7 (0.3)	22.0 (1.8)[i]
	20	6.9 (0.5)[f]	22.5 (1.5)[g]	6.4 (0.3)	20.8 (1.0)[i]
Glycated hemoglobin (% total hemoglobin)	Sacrifice	4.08 (0.14)[c]	9.93 (0.36)[g]	3.05 (0.05)	10.10 (0.34)[i]

[a] Data are presented as mean (SEM). Control-diabetic, control-nondiabetic, transgenic-diabetic, and transgenic-nondiabetic groups are denoted CON-D, CON-ND, TG-D, and TG-ND, respectively. Urine was collected over a 23-h period except for control rats at 9 wk (18-h collection). Rats were sacrificed after 22 wk of diabetes.
[b] $p < 0.01$ vs value at wk 20 for same group.
[c] $p < 0.01$ for CON-ND vs TG-ND.
[d] $p < 0.05$ for CON-ND vs TG-ND.
[e] $p < 0.01$ vs value at wk 9 for same group.
[f] $p < 0.05$ vs value at wk 9 for same group.
[g] $p < 0.01$ for CON-D vs CON-ND.
[h] $p < 0.05$ vs value at wk 16 for same group.
[i] $p < 0.01$ for TG-D vs TG-ND.

tion) and 28 wk of diabetes (long duration) (data not shown). This suggested that alterations in GFR were not likely to underlie the reduction in albuminuria; nevertheless, this conclusion was drawn cautiously since the result was obtained from a separate cohort.

The urinary excretion of two protein markers of tubular function, β_2-microglobulin and *N*-acetyl-β-D-glucosaminidase, was also determined. At 20 wk of diabetes, when the effect of overexpression of aldose reductase on albuminuria was most pronounced, excretion of both proteins was significantly higher in both diabetic groups compared with nondiabetic counterparts, and there was no effect of the transgene on these parameters (Table 2).

Discussion

The main body of evidence for a pathogenic role for aldose reductase in diabetic nephropathy derives from aldose reductase enzyme inhibition studies *(16)*. However, the known nonspecific properties of these inhibitors preclude a firm conclusion from being drawn *(12–15)*. Alternative approaches that complement inhibitor studies are therefore necessary to establish the role of aldose reductase in diabetic nephropathy.

One such approach is through the use of transgenic animal models that overexpress aldose reductase. Prior to the present study, others had developed a transgenic mouse model (denoted hAR-Tg) overexpressing the human enzyme *(17)*. In these animals, galactose feeding, rather than induction of experimental diabetes, was used to study the consequences of overexpression of aldose reductase. Galactosemia resulted in the presence of thrombi in renal vessels and fibrinous deposits in the Bowman's capsule of the glomeruli in hAR-Tg mice but not in nontransgenic littermates *(17)*. Overexpression of aldose reductase did not exert any significant impact on albuminuria in galactose-fed hAR-Tg mice com-

Table 2
Effect of STZ Diabetes on Renal Parameters in Cohorts of Control and Homozygous TgCMVhAR$_2$ Transgenic Rats[a]

Renal parameter	Time after diabetes onset (wk)	CON-ND (n = 6)	CON-D (n = 6)	TG-ND (n = 6)	TG-D (n = 5)
Albumin (μg/h)	9	37.6 (3.8)[c]	103.2 (24.8)[c,e]	47.2 (7.9)	58.2 (9.3)[f]
	12	54.9 (6.1)[c]	162.9 (69.1)[f]	56.4 (5.8)	60.5 (11.3)[f]
	16	75.5 (9.1)[c]	210.7 (66.0)	82.3 (15.3)	106.6 (21.0)
	20	178.9 (27.8)[d]	459.2 (90.9)[e,g]	86.2 (15.8)	179.1 (49.1)
β_2-Microglobulin (μg/h)	20	0.23 (0.02)	2.54 (0.40)[h]	0.32 (0.02)	3.33 (0.66)[j]
N-acetyl-β-D-glucosaminidase (mU/h)	20	6.51 (0.33)	23.11 (1.82)[h]	6.36 (0.29)	19.81 (4.08)[j]
Kidney mass (g)	Sacrifice	1.86 (0.09)	2.46 (0.14)[h]	1.82 (0.10)	2.26 (0.06)[j]
Kidney/body mass (%)	Sacrifice	0.42 (0.02)	0.73 (0.06)[h]	0.41 (0.02)	0.73 (0.02)[j]
Glomerular tuft area (μm^2)[b]	Sacrifice	8450 (351)	10,480 (509)[i]	9360 (579)	11,780 (722)[j]
Bowman's area (μm^2)[b]	Sacrifice	3450 (270)	4120 (326)	3340 (237)	4490 (344)[j]
Glomerular tuft area/ Bowman's area ratio[b]	Sacrifice	2.70 (0.21)	2.80 (0.22)	2.93 (0.22)	2.91 (0.18)
Tubular cross-sectional area (m^2)[b]	Sacrifice	615 (58)	1135 (70)[i]	625 (62)	1142 (70)[j]

[a] Data are presented as mean (SEM). Control-diabetic, control-nondiabetic, transgenic-diabetic, and transgenic-nondiabetic groups are denoted CON-D, CON-ND, TG-D, and TG-ND, respectively. Urine was collected over a 23-h period except for control rats at 9 wk (18-h collection). Rats were sacrificed after 22 wk of diabetes.
[b] Morphometric parameters calculated from n = 20 images.
[c] $p < 0.01$ vs value at wk 20 for same group.
[d] $p < 0.05$ for CON-ND vs TG-ND.
[e] $p < 0.05$ for CON-D vs CON-ND.
[f] $p < 0.05$ vs value at wk 20 for same group.
[g] $p < 0.05$ for CON-D vs TG-D.
[h] $p < 0.01$ for CON-D vs CON-ND.
[i] $p < 0.005$ for CON-D vs CON-ND.
[j] $p < 0.01$ for TG-D vs TG-ND.

pared with their nontransgenic counterparts *(18)*. This negative finding was somewhat unexpected considering that ARI treatment was able to prevent galactosemia-induced albuminuria in the same study *(18)*.

The effect of induction of diabetes on kidney function and structure has not yet been reported for the hAR-Tg mice. Although dietary galactosemia may be suitable for the study of diabetic cataractogenesis and retinopathy *(19,20)*, the utility of this dietary model for the study of diabetic nephropathy is questionable. Specifically, while an increase in GFR, renal plasma flow, and glomerular basement membrane thickening has been reported in both alloxan diabetic and galactosemic dogs *(21,22)*, galactose feeding did not precipitate other renal changes that characterize diabetic nephropathy *(21)*. In galactosemic mice and rats, the profile of urinary albumin excretion with respect to time also differed from diabetic counterparts *(18,23)*. In all, galactosemia-induced alterations in renal functional and structural parameters exhibit discordance from those caused by diabetes. Accordingly, these considerations also prevent any direct comparison between the phenotypes of the galactose-fed hAR-Tg mice and the STZ diabetic TgCMVhAR$_2$ rats in our study.

Contrary to what may be anticipated from the results of ARI studies, overexpression of aldose reductase resulted in a marked reduction in albuminuria in diabetic rats. At present, the precise mechanism underlying this apparently protective phenomenon is not obvious. This is especially so, considering that all current hypotheses have implicated aldose reductase in a solely pathogenic capacity *(16)*. The solution is further complicated by the proposed convergence between the role of aldose reductase with that of other pathogenic pathways including protein kinase C activation, oxidative stress, and advanced glycation product (AGE) formation *(24)*.

However, we have considered the possibility that in TgCMVhAR$_2$ rats, aldose reductase might decrease albuminuria by alleviating carbonyl stress, the level of which is raised by diabetes *(25–30)*. This hypothesis is based on the known ability of aldose reductase to chemically reduce hydrophilic and hydrophobic aldehydes to their alcohols *(31–41)*. Preliminary in vitro studies appeared to support

this notion. Briefly, we have previously shown that the amount of protein-bound carbonyls in S3 segments of renal proximal tubules from nondiabetic $TgCMVhAR_2$ rats is significantly lower than that of control rats (2.9 ± 0.30 vs 4.0 ± 0.22 nmol of protein-bound dicarbonyl/mg of protein; $n = 6$, $p < 0.01$) *(42)*. This effect of overexpression of aldose reductase on decreasing protein-bound carbonyl accumulation could be prevented by enzyme inhibition with an ARI *(42)*. In this context, we observed that despite highly comparable blood glucose control, there was clearly a significant reduction in age-dependent albuminuria in nondiabetic transgenic rats compared with controls, and this was accompanied by a 25% decrease in glycated hemoglobin content. The latter finding suggests that alleviation of carbonyl stress by overexpression of aldose reductase might extend beyond the confines of transgene-expressing cells/tissues.

Since diabetes-associated changes in the glomerulus may contribute to albuminuria and since aldose reductase has been implicated in some of these glomerular alterations *(10,11)*, a limitation of the present study is related to the apparent absence of transgene expression in glomerulus. This absence occurred despite the use of the CMV promoter, a regulatory DNA sequence previously widely regarded to be constitutive in its activity. This transgenic model will not be informative in revealing the role of aldose reductase at a glomerular location. Instead, it serves to strongly highlight the potential contribution made by aldose reductase to renal tubular function. Supporting this contention, albuminuria was prevented by transgene expression in the absence of any major changes in glomerular structure, as indicated by measurements of the glomerular tuft area and Bowman's area. With respect to this finding, it is relevant to note that in a recent study by Ziyadeh et al. *(43)*, antibody-mediated neutralization of transforming growth factor-β (TGF-β), a cytokine implicated as a key mediator in diabetic nephropathy, prevented diabetes-induced glomerular mesangial changes, yet had no effect on diabetes-induced albuminuria. Despite the possible corroboration of our results by this TGF-β neutralization study, it would be premature to exclude a role for aldose reductase in structural and functional changes seen in the glomerulus in diabetes.

Albumin excretion is affected by glomerular filtration and subsequent renal tubular handling. Morphologic and functional studies that have examined renal tubular handling of filtered albumin have demonstrated that reabsorption of filtered albumin occurs primarily at the proximal tubule by endocytosis. Consequently, examination of the impact of aldose reductase overexpression in the renal proximal tubules may prove highly relevant in aiding our understanding of the pathogenesis of diabetic nephropathy *(44)*. Indeed, it has been demonstrated that within the kidney, the renal proximal tubules represent the major site for the accumulation of AGEs *(45–47)*, and diabetes-associated AGE accumulation in this renal location may be linked to pathogenic mechanisms that contribute to diabetic nephropathy *(44,47)*. In addition to these observations, the proximal tubule is particularly susceptible to iron overload–induced carbonyl stress as assessed by 4-hydroxynonenal modified protein accumulation *(48)*. Pharmacologic agents that act to scavenge 4-hydroxynonenal and other reactive carbonyl molecules may thus be expected to ameliorate diabetes-induced albuminuria. These properties are present in some currently used modifiers of diabetic renal complications (e.g., aminoguanidine, an inhibitor of AGE formation *[49–57]*). The beneficial effects on albuminuria seen with aminoguanidine *(58–61)* could be related in part to a diminution of carbonyl stress at the renal proximal tubules, which are actively involved in albumin reabsorption.

To characterize further the effect of overexpression of aldose reductase on renal function, we determined the excretion of two commonly used indicators of renal proximal tubular dysfunction, β_2-microglobulin and *N*-acetyl-β-D-glucosaminidase. Despite their utility, the results of these experiments should be interpreted critically since increased excretion of these proteins may only reflect dysfunction at specific proximal tubular segments. Specifically, decreased β_2-microglobulin uptake at the proximal tubule most likely signifies damage at the late S1 and S2 segments since these are the predominant sites in the rat kidney where the transmembrane protein megalin serves as the cell-surface receptor for β_2-microglobulin (among other ligands including albumin) *(62,63)*. For *N*-acetyl-β-D-glucosaminidase, the appearance of this protein in the urine derives from tubular turnover/damage, primarily in the proximal convoluted (S1) tubule segment *(64)*. By contrast, albumin reabsorption capability is evenly distributed among the S1, S2, and S3 segments *(65)*. In addition, the S3 segment is capable of further albumin uptake in the event of albumin overload *(65)*. The observation that while overexpression of aldose reductase normalized the urinary excretion of albumin but not β_2-microglobulin and *N*-acetyl-β-D-glucosaminidase would be compatible with transgene expression at the S3 segment of the proximal tubule.

In conclusion, the present study raises the possibility of a protective effect of renal tubular aldose reductase in diabetic tubulopathy. Future experiments will be essential to determine the complex (protective and detrimental) role of aldose reductase as well as the interplay of this enzyme with other pathogenic mechanisms implicated in diabetic nephropathy.

Materials and Methods

Animal Ethics

All protocols involving the use of animals were approved by the Animal Ethics Committee of the Royal Melbourne Hospital Research Foundation and conformed to the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (www.nhmrc.health.gov.au/research/awc/code.htm).

Animal Husbandry

All rats used were of the PVG/c strain. Rats were housed at the Centre for Animal Biotechnology (Veterinary Precinct, University of Melbourne). The room was maintained at 20ºC and a 12-h night/day cycle with lights on at 7:00 AM was imposed. Rats (caged in groups of three) were allowed free access to normal rat chow (Barastoc Stockfeeds, Victoria, Australia) and autoclaved tap water. For urine collections, rats were housed singly with free access to food and water in metabolic cages (Iffa Credo, L'Abresele, France). When required, rats were euthanized by gassing with carbon dioxide.

Generation of Transgenic Rats

The linearized CMVhAR$_2$ transgene (4.0 kb) was excised from the plasmid pCMVhAR$_2$ *(66)* following digestion with *Pvu*II. It consisted mainly of the CMV promoter joined to a human placental aldose reductase cDNA *(67)*, which was in turn coupled to the SV40 splice and polyadenylation signal sequence (Fig. 1). Pronuclear microinjection of fertilized one-cell oocytes was performed according to the method of Brinster et al. *(68)*. Fertilized PVG/c rat oocytes were harvested from the oviducts of sexually immature female rats that had been superovulated using 20 IU of pregnant mare's serum gonadotropin and 10 IU of human chorionic gonadotropin (Intervet, New South Wales, Australia) and then mated. Successfully microinjected oocytes were introduced into the oviducts of surrogate pseudopregnant female rats, with live births occurring 3 wk later.

Induction of STZ Diabetes

Diabetes was induced in male rats (200 g fasted body wt) by an ip injection of STZ (65 mg/kg body wt) in 100 m*M* sodium citrate buffer (pH 4.0). These rats were allowed access to sucrose (5%) in drinking water for the next 24 h to prevent hypoglycemia. Successful induction of diabetes was demonstrated within 3 d after STZ injection by glycosuria. For long-term diabetic rats, isophane insulin (Protaphane®; Novo Nordisk) was administered at a dose of 2 U per rat subcutaneously, for a total of three times a week. Insulin treatment was commenced 2 wk after onset of diabetes.

Characterization of Transgenic Cohorts

Genomic Southern Analysis

Transgenic rats were identified by genomic Southern analysis. Rat tail biopsies were digested overnight with proteinase K (Boehringer Mannheim, New South Wales, Australia) at 55ºC and then RNase treated (Boehringer Mannheim) for 2 h. This was followed by phenol:chloroform extraction and precipitation with absolute ethanol. After washing, the DNA pellet was dissolved in TE buffer. Genomic DNA was digested with *Bam*HI or other appropriate restriction enzymes (Promega, Madison, WI). Digested samples were separated in an agarose gel and transferred onto a nylon membrane filter (Amersham International, Buckinghamshire, England). The filter was prehybridized at 65ºC before addition of denatured transgene DNA probe (Fig. 1) labeled with [α-^{32}phosphorus]-dATP (Bresatec, South Australia, Australia) using the Prime-a-Gene® System (Promega). After washing, the presence of bound radioactive signal was detected using autoradiographic film (Eastman Kodak).

Northern Analysis

Total RNA was extracted from both rat tissues and cultured cells using an acid guanidinium thiocyanate-phenol-chloroform method *(69)*. Total RNA was fractionated on a denaturing formaldehyde agarose gel and then transferred to nylon membrane using 20X saline sodium citrate (SSC). Hybridization and probe labeling were carried out as with genomic Southern analysis (Fig. 1). The filter was then washed and subjected to autoradiography. RNA loading was normalized against endogenous rat GAPDH mRNA.

In situ RNA Hybridization

Freshly dissected tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 18–24 h at room temperature before being embedded in paraffin wax and cut into 5-µm-thick sections. These were dewaxed, rehydrated, and treated with pronase E (125 g/mL) before being rinsed in 0.1 *M* sodium phosphate buffer (pH 7.2), postfixed in 4% paraformaldehyde/PBS, followed by rinsing in 0.1 *M* sodium phosphate buffer and deionized water. The sections were dehydrated in 70% ethanol and then left to dry.

Sense and antisense riboprobes (RNA probes) were prepared by cloning the relevant DNA fragments into the plasmid pGEM-5Zf(+) (Promega), followed by in vitro transcription in the presence of [α-^{35}sulfur]-CTP (Bresatec) using the Riboprobe® System (Promega) (Fig. 1). *In situ* RNA hybridization was carried out in a sealed box, humidified with 50% formamide. The slides were washed in 50% formamide/2X SSC solution, treated with RNase A (150 µg/mL), and then washed in 2X SSC. Dehydrated sections were coated in photographic emulsion (Ilford K5) and exposed for 7–21 d before developing. Sections were also stained in Mayer's hematoxylin and dehydrated.

Western Blot Analysis

Fresh kidneys were placed in cold Dulbecco's modified Eagle's medium before being cut into longitudinal or transverse sections as required. Kidneys were rendered free of all surrounding fat and membranous tissues, and when appropriate, the renal papillae were also removed. Each kidney section was homogenized completely in protein lysis buffer and subjected to three cycles of freeze thawing before being placed on a rotating wheel at 4ºC for 1 h. The homogenate was then centrifuged at 15,000*g* for 5 min. The supernatant was kept at –70ºC until analysis. Protein concentration was determined using a commercially available protein assay (Bio-Rad, Hercules, CA).

Protein samples were fractionated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide resolving gel using

SDS-polyacrylamide gel electrophoresis running buffer. Gel contents were electroblotted onto a piece of Polyscreen® polyvinyl difluoride transfer membrane (NEN Life Sciences). For Western blot detection of both human and rat endogenous aldose reductase, the membrane was incubated in 5% (w/v) skim milk powder solution at 4ºC overnight. Rabbit polyclonal antibodies raised against human aldose reductase *(70)* (1:10,000 dilution in 5% [w/v] skim milk powder solution) were added and left overnight at 4ºC. Next, the membrane was rinsed in TBS buffer before incubation with horseradish peroxidase–conjugated goat anti-rabbit IgG (Dako, New South Wales, Australia) (1:2000 dilution in 5% [w/v] skim milk powder solution) overnight at 4ºC. The membrane was then washed in TBS (with 0.05% Tween-20). Enhanced chemiluminescence detection was achieved using the Renaissance® Western Blot Chemiluminescence reagent (NEN Life Sciences) together with Hyperfilm® MP autoradiography film (Amersham International).

Determination of Blood Glucose

Blood glucose levels from rat tail bleeds were determined using the Precision Q•I•D® blood glucose sensor with Medisense®2 blood glucose sensor electrodes (Medisense Australia, Victoria, Australia).

Glycated Hemoglobin Analysis

Blood from rat tail bleeds was collected in EDTA-treated tubes at the time of sacrifice and sent to the Austin and Repatriation Medical Centre (Victoria, Australia) for determination of glycated hemoglobin *(71)*. The quantity of glycated hemoglobin present was expressed as a percentage of total hemoglobin.

Measurement of Urinary Excretion of Albumin, β_2-Microglobulin, and N-acetyl-β-D-glucosaminidase

Urinary albumin determination was performed using a commercially available enzyme immunoassay kit for rat albumin (SPI-BIO, France). β_2-Microglobulin excretion was measured using a β_2-micro radioimmunoassay kit (Pharmacia AB, Uppsala, Sweden). Intraassay coefficient of variation (CV) was determined to be 0.04. *N*-acetyl-β-D-glucosaminidase excretion was evaluated using a commercially available colorimetric assay (Boehringer Mannheim). Intraassay CV was determined to be 0.04. Urine samples assayed were pH 6.0, as assessed by pH indicator paper.

Morphometric Study

Rats were anesthetized with Nembutal (40–60 mg/kg body wt) (Boehringer Ingelheim, Artamo, Australia). Following an abdominal midline incision, the abdominal aorta and vena cava were exposed, the abdominal aorta was cannulated, and kidneys were perfusion fixed by retrograde perfusion of Karnovsky's fixative following an established protocol *(72)*. Kidney sections (2 µm thick) were stained with toluidine blue and periodic acid-Schiff for light microscopy, photography, and quantification. The planar surface area of all glomeruli in six photographic fields (>25 glomeruli for each condition) was inscribed and quantified. Subsequently, the glomerular tuft area was inscribed and Bowman's area derived by subtraction. Tubular morphology was assessed visually, and luminal diameter was calculated from photographic images without reference to tubular subdivision or locality within the kidney section (predominantly cortex and outer stripe of outer medulla).

Statistical Analysis

Group data are presented as the mean ± SEM. For multiple comparisons, one-way analysis of variance was used to determine differences among all treatment groups. Statistical comparisons between two treatment groups were performed using student's *t*-test (two-tailed) for unpaired samples. Statistical significance was taken at the 5% level unless otherwise indicated.

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