

Genetic deficiency of aldose reductase counteracts the development of diabetic nephropathy in C57BL/6 mice

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Abstract

Aims/hypothesis The aim of the study was to investigate the effects of genetic deficiency of aldose reductase in mice on the development of key endpoints of diabetic nephropathy. **Methods** A line of *Ar* (also known as *Akr1b3*)-knockout (KO) mice, a line of *Ar*-bitransgenic mice and control C57BL/6 mice were used in the study. The KO and bitransgenic mice were deficient for *Ar* in the renal glomeruli and all other tissues, with the exception of, in

the bitransgenic mice, a human *AR* cDNA knockin-transgene that directed collecting-tubule epithelial-cell-specific *AR* expression. Diabetes was induced in 8-week-old male mice with streptozotocin. Mice were further maintained for 17 weeks then killed. A number of serum and urinary variables were determined for these 25-week-old mice. Periodic acid–Schiff staining, western blots, immunohistochemistry and protein kinase C (PKC) activity assays were performed for histological analyses, and to determine the levels of collagen IV and TGF- β 1 and PKC activities in renal cortical tissues.

Results Diabetes-induced extracellular matrix accumulation and collagen IV overproduction were completely prevented in diabetic *Ar*-KO and bitransgenic mice. *Ar* deficiency also completely or partially prevented diabetes-induced activation of renal cortical PKC, TGF- β 1 and glomerular hypertrophy. Loss of *Ar* results in a 43% reduction in urine albumin excretion in the diabetic *Ar*-KO mice and a 48% reduction in the diabetic bitransgenic mice ($p < 0.01$).

Conclusions/interpretation Genetic deficiency of *Ar* significantly ameliorated development of key endpoints linked with early diabetic nephropathy in vivo. Robust and specific inhibition of aldose reductase might be an effective strategy for the prevention and treatment of diabetic nephropathy.

Keywords Aldose reductase · Collagen IV · Diabetic nephropathy · Oxidative stress · PKC · Polyol pathway · TGF- β 1 · Urinary albumin

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Abbreviations

AR Aldose reductase
ARI Aldose reductase inhibitor
BT $AR^{-/-}$ $KspAR^{+/-}$ bitransgenic
BUN Blood urea nitrogen

Cit	Citrate buffer
DN	Diabetic nephropathy
ECM	Extracellular matrix
GSH	Glutathione
KO	Knockout
MDA	Malondialdehyde
PAS	Periodic acid–Schiff
PKC	Protein kinase C
SOD	Superoxide dismutase
STZ	Streptozotocin
UAE	Urinary albumin excretion
WT	Wild type

Introduction

In the mammalian kidneys, aldose reductase (AR) is abundantly produced in the inner medulla, where it converts glucose to sorbitol, the latter serves as a critical organic osmolyte to protect the collecting duct tubule cells from hyperosmotic insults [1] (the human gene encoding AR is *AKR1B1* [also known as *AR*] and the mouse gene is *Akr1b3* [also known as *Ar*]). In contrast to its prominent expression in the renal medulla, AR production in the renal cortex is low to moderate, depending on cell types. Under normal physiological conditions, AR production in the glomerulus is low. However, a significant increase in glomerular AR production was found in diabetic human patients [2, 3]. Diabetes in rats led to a tenfold increase in glomerular sorbitol content, whereas sorbitol was unchanged in diabetic rats treated with an aldose reductase inhibitor (ARI), sorbinil [4]. Within the glomerulus, *Ar* mRNA expression is strongly induced in the mesangial cells [5, 6] and the endothelial cells [7] under hypertonic conditions. Overactivation of AR in renal cells has been linked with aberrant activation of protein kinase C (PKC) [8–10], generation of advanced glycation products, increased expression of TGF- β and generation of reactive oxygen species [11]. Together these data suggest that significant activation of AR by hyperglycaemia in the renal glomeruli might contribute to the onset or progression of glomerulopathy and diabetic nephropathy (DN).

AR is the first and the rate-limiting enzyme of the polyol pathway, a pathway that has been implicated in the development of diabetic complications, particularly diabetic retinopathy and diabetic neuropathy [11, 12]. The association of AR and the polyol pathway with the development of DN, however, is less conclusive, partly because the data obtained from genetic, biochemical, pharmacological, animal and clinical studies have been less consistent [13]. For instance, some human genetic studies support the association of *AR* 5'-end polymorphism with DN [14–19] while

others did not confirm this [19–21]. In one animal model study, transgenic overexpression of human *AR* in mice led to pathological changes in the kidney, lens and retina [22]. In another communication, however, it was reported that diabetes-induced albuminuria was prevented in transgenic rats overexpressing human *AR* selectively in the straight (S3) portion of renal proximal tubules [23]. Most pharmacological experiments demonstrated a positive influence of chemically synthesised ARIs on the development of DN in diabetic rats [13, 24–26], but a few failed [27, 28]. Further, although some human clinical trials have reported unchanged glomerular function after ARI treatment [29], encouraging results have also been obtained that clearly demonstrate the beneficial effects of the inhibition of AR with ARIs [13, 30].

The reasons for the inconsistency with regard to the pathophysiological roles of AR and the effectiveness of ARIs in DN are not completely clear. However, incomplete AR inhibition, the presence of multiple AR-like proteins, individual genetic differences and the specificity, side or toxic effects of ARIs are among the possible contributing factors. Studies have shown that human and rodent kidney tissues possess a number of AR-like proteins, such as AR-like gene-1 [31] and renal-specific oxidoreductase [32], as well as renal aldehyde reductases [33]. It is not clear how these structurally and functionally similar proteins might interact in vitro and in vivo. Meanwhile, the specificity of these inhibitors used in animal studies or clinical studies remains open to question. Some of them are already known to be able to inhibit aldehyde reductases, in addition to AR [33]. Such 'off-target' effects might underlie undesired side effects or even toxic effects.

One approach to overcome the shortcomings associated with the use of ARIs is to use a gene-knockout (KO) model. Mice globally deficient in *Ar* (*Ar*^{-/-}), however, were found to carry defects in the renal medulla characterised by renal medullary atrophy and epithelial cell death [34, 35]. As a consequence of these medullary lesions, *Ar*-deficient mice develop a phenotype resembling that of nephrogenic diabetes insipidus in human [34, 35]. Because of this problem, no DN study has yet been performed with *Ar*-deficient mice, even though they have been available for more than 10 years [34, 36]; therefore, the data from a gene-knockout model have been lacking. Recently, we created a double-transgenic mouse line (*Ar*^{-/-} *KspAR*^{+/-} bitransgenic [BT]) that is deficient in AR in all tissues and the kidney glomeruli except in the inner medulla, where an *AR*-knockin transgene is driven by the kidney-specific cadherin promoter to direct *AR* expression specifically in the collecting tubule epithelial cells [35]. The BT mice differ from the *Ar*-KO mice in that the *Ar*-KO mice are completely deficient in AR whereas the BT mice are deficient in AR in all tissues, including most parts of the

kidney, except the medullary collecting tubule epithelial cells that carry the knockin transgene. The glomeruli from the *Ar*-KO and BT mice, however, are equivalent in terms of AR production. As a consequence of the medullary epithelial cell-specific *AR* expression of the knockin transgene, the cellular lesions in renal medulla and the urine concentrating mechanisms were shown to be significantly corrected in the BT mice [35]. In our current study, we used the *Ar*-KO and BT mice to investigate the effects of genetic deficiency of *Ar* on the development of early DN. Our results indicate that genetic ablation of *Ar* significantly ameliorates the development of DN in streptozotocin (STZ)-induced diabetic C57BL/6 mice.

Methods

Animals and animal treatments All animal experiments were carried out in accordance with the guidelines of the Xiamen University Institutional Committee for the Care and Use of Laboratory Animals. *Ar*^{-/-} mice [34] were prepared and backcrossed with C57BL/6 mice for seven generations. A line of transgenic mice (*KspAR*^{+/-}) in which a human *AR* transgene driven by the mouse kidney-specific cadherin promoter to overproduce AR specifically in the collecting tubule epithelial cells was generated and backcrossed with C57BL/6 mice for six generations as described [35]. Subsequent intercrosses between the KO mice and the *KspAR*^{+/-} mice generated the BT mice heterozygous for *KspAR* and homozygous for the *Ar*-KO allele (*Ar*^{-/-}*KspAR*^{+/-}), as well as the *Ar*-KO mice and the wild-type (WT) mice [35]. The genotypes of three groups of mice were verified as described previously by Yang et al. [35]. The KO, BT and WT mice were, therefore, relatively homogeneous for the C57BL/6 background that was maintained as described previously [35]. For each particular genotype, male mice, 8 weeks old, were randomly divided into two subgroups (each having at least six mice): one was intraperitoneally injected with 40 mg kg⁻¹ day⁻¹ for five consecutive days; and the other was injected with a vehicle solution (0.1 mol/l citrate buffer [Cit]). Immediately after the diabetic induction, blood glucose was assayed with a Glucometer (OneTouch Ultra, LifeScan, Milpitas, CA, USA) and only those mice with blood glucose levels above 16 mmol/l were considered to be diabetic. Six treatment groups of mice were therefore generated. They were: non-diabetic WT (WT + Cit) mice; non-diabetic KO (KO + Cit) mice; non-diabetic BT (BT + Cit) mice; diabetic WT (WT + STZ) mice; diabetic KO (KO + STZ) mice; and diabetic BT (BT + STZ) mice. The diabetic and non-diabetic mice were maintained for an additional 17 weeks. The animals were transferred to metabolism cages 1 day before the age of 25 weeks (one mouse/cage, with free access to standard

chow diet and water) for 24 h for urine sample collection. Blood samples were collected by sinus puncture and the animals were subsequently killed and kidneys were dissected for analyses.

PKC activity assays PKC activity assays were carried out with the PepTag Nonradioactive PKC Assay System from Promega (Beijing, China) according to the manufacturer's instructions. Briefly, renal cortical tissues were homogenised in 2 ml ice-cold extraction buffer (supplied) with a Kinematica homogeniser (Lucerne, Switzerland). The homogenates were centrifuged at 100,000×g and 4°C for 60 min. The supernatant fractions were taken as the cytosol fractions whereas the pellets were further dissolved in 2 ml of the extraction buffer with 1% Triton X-100. After 30 min incubation at 4°C, the solutions were centrifuged again at 100,000×g for 60 min, and the second supernatant fractions were used as the membrane fractions. The concentrations of protein in the preparations were determined with the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The membrane and cytoplasmic fractions were then used for PKC activity assays, following the manufacturer's instructions. The phosphorylated and non-phosphorylated reaction mixtures were separated on a 0.8% agarose gel. The gels were visualised under UV light and the bands were quantified by densitometric analysis using Image-Pro Plus software (Media Cybernetics, Shanghai, China).

Serum analyses, urine analyses, western blots for TGF-β1 and collagen IV, immunohistochemistry for renal expression of collagen IV and other renal histological analyses These experimental procedures are described in detail in the Electronic supplementary material (ESM).

Statistical analyses All statistical analyses were performed with the GraphPad Prism. Values are expressed as the means ± SEM. Multiple group comparisons were performed with one-way ANOVA with the Bonferroni's post test and pair-wise comparisons were performed with unpaired Student's *t* test. A probability value <0.05 was considered to be significant.

Results

***AR* deficiency in the *Ar*-KO and BT mice significantly ameliorated diabetes-induced renal hypertrophy, elevations in serum triacylglycerols and serum LDL-cholesterol and urinary albumin excretion (UAE)** To assess the effects of genetic deficiency of *Ar* on the development of DN, we treated the *Ar*-KO, BT and WT C57BL/6 mice with multiple low doses of STZ (40 mg/kg, i.p. for five

consecutive days) to destroy the mouse pancreatic beta cells and to induce diabetes. After STZ injection, blood glucose levels were determined and only those with blood glucose levels greater than or equal to 16 mmol/l were considered to be diabetic. Both the STZ-treated mice and the control mice were further maintained for 17 weeks under normal rearing conditions (free access to standard chow and water) to allow the development of diabetic kidney diseases.

At the age of 25 weeks (17 weeks from diabetes onset), diabetic WT mice exhibited typical pathophysiological features of early DN (Table 1). With the exception of serum HDL-cholesterol, the body weight, serum content of triacylglycerol, total cholesterol, LDL-cholesterol, blood urea nitrogen (BUN), GFR and UAE were all significantly altered in diabetic WT mice, manifesting as the development of diabetes or early DN. In the diabetic *Ar*-KO mice, complete deficiency of AR appeared to result in significant improvement in the ratio of kidney/body weight (6.80 ± 0.52 for diabetic *Ar*-KO mice vs 8.20 ± 0.45 for diabetic WT mice, $p < 0.05$), serum triacylglycerols (0.57 ± 0.07 mmol/l for diabetic *Ar*-KO mice vs 1.11 ± 0.18 mmol/l for diabetic WT mice, $p < 0.001$), serum LDL-cholesterol (1.16 ± 0.13 mmol/l for diabetic *Ar*-KO mice vs 1.62 ± 0.13 mmol/l for diabetic WT mice, $p < 0.05$) and UAE (1.49 ± 0.26 µg/mg creatinine for diabetic *Ar*-KO mice vs 2.62 ± 0.34 µg/mg creatinine for diabetic WT mice, $p < 0.001$). In contrast to these DN-associated variables, the BUN and GFR were not significantly improved in the diabetic *Ar*-KO mice. However, in diabetic BT mice, not only the body weight, but the ratio of kidney/body weight, serum triacylglycerols and LDL-cholesterol, and UAE were all significantly improved compared with the diabetic WT mice; BUN (6.28 ± 0.19 mmol/l for diabetic BT mice vs 8.75 ± 0.53 mmol/l for diabetic WT mice, $p < 0.001$) and GFR (0.18 ± 0.01 l/24 h for diabetic BT mice vs 0.12 ± 0.01 l/24 h for diabetic WT mice, $p < 0.05$) both also appeared to be significantly improved. In the STZ-treated groups, the three genotypes of diabetic mice all excreted large volumes of urine that were not statistically different. As these volumes of urine were much larger than those in AR-deficiency-induced polyuria, it suggests that hyperglycaemia is the predominant contributing factor for the polyuric phenotype. Together, these biochemical analyses indicate that AR deficiency resulted in improvement of most serum and urinary variables associated with DN in diabetic C57BL/6 mice.

AR deficiency in the diabetic Ar-KO and BT mice significantly ameliorated diabetes-induced glomerular hypertrophy, cell proliferation, mesangial expansion and collagen IV expression To examine for potential renal cortical morphological differences in the diabetic WT mice and the AR-deficient diabetic mice, we performed periodic acid–Schiff (PAS) staining with the renal cortical tissues

dissected from six treatment groups of mice at the age of 25 weeks. As shown in Fig. 1a, no apparent difference in glomerular morphology was observed for three non-diabetic genotype groups of mice. The glomeruli were of normal size and configuration for non-diabetic WT, *Ar*-KO and BT mice. Further, there was no sign of mesangial matrix expansion, inflammation or sclerosis in these mice. In contrast to the findings in these non-diabetic mice, very significant glomerular hypertrophy, increased cellularity and narrowing of capillary lumens was observed in the cortex/glomeruli of diabetic WT mice (Fig. 1a). Further, the mesangium was diffusively and markedly expanded with PAS-positive (purple colour) matrix material. When the digital images of about 30 glomeruli for each genotype were quantitatively analysed, the extracellular matrix (ECM) accumulation index for the diabetic WT mice was, on average, 41% higher than that for the non-diabetic WT mice (0.099 ± 0.002 for diabetic WT vs 0.070 ± 0.003 for non-diabetic WT, $p < 0.01$, Fig. 1b), whereas the glomerular size for the diabetic WT on average was 68% higher than that for the non-diabetic WT mice ($1,733 \pm 13.9$ for diabetic WT vs $1,031 \pm 36.7$ for non-diabetic WT, $p < 0.001$, Fig. 1c). These results indicate significant lesions in renal cortical/glomerular structures in the WT diabetic mice at the age of 25 weeks. Interestingly, in the glomeruli of diabetic *Ar*-KO and BT mice, diabetes-induced glomerular hypertrophy, cell proliferation and mesangial matrix expansion were all significantly corrected (Fig. 1a–c). Despite the exposure to hyperglycaemia for 17 weeks, glomerular size and ECM accumulation and configuration in the diabetic *Ar*-KO and BT mice were almost indistinguishable from those in the non-diabetic mice, characterised by improved glomerular hypertrophy, reduced cellularity and normalisation of mesangial expansion (Fig. 1a–c). These results indicated significant restoration in the renal cortical/glomerular structures as a consequence of genetic deficiency of *Ar*.

In contrast to the significant differences in the cortex/glomeruli areas, the outer medullary areas did not show apparent histomorphological differences among genotype groups of mice as determined by PAS staining (ESM Fig. 1). In the inner medulla, however, severe tubular atrophy was observed for all three genotype groups of diabetic mice. The tubulointerstitial lesions in the diabetic BT mice, however, appeared to be slightly improved (ESM Fig. 2) as compared with the diabetic *Ar*-KO mice.

As collagen IV is the principal protein of the expanded ECM in the diabetic renal glomeruli [37, 38], we analysed the production of collagen IV by immunohistochemistry and western blot. As shown in Fig. 2, in non-diabetic WT mice, relatively low collagen IV production was found in the mesangial cells, the Bowman's capsule and the tubule basement membranes. In strong contrast to this, very prominent collagen IV production was found in the cortical

Table 1 Physiological variables of the six mouse groups at the age of 25 weeks

Variable	Treatment groups						<i>p</i> values			
	Non-diabetic WT + Cit	Non-diabetic KO + Cit	Non-diabetic BT + Cit	Diabetic WT + STZ	Diabetic KO + STZ	Diabetic BT + STZ	WT + STZ vs WT + Cit	KO + STZ vs WT + STZ	BT + STZ vs WT + STZ	
Body weight (g)	30.98±0.93	29.88±1.52	29.87±1.06	22.48±1.03	21.88±1.74	26.65±0.72	<0.001	NS	<0.05	
Kidney weight/body weight ($\times 10^3$)	4.94±0.13	4.47±0.11	5.00±0.08	8.20±0.45	6.80±0.52	6.82±0.28	<0.001	<0.05	<0.05	
Serum glucose (mmol/l)	7.72±0.65	8.12±0.48	8.32±0.27	35.37±0.63	32.47±2.12	26.27±0.87	<0.001	NS	<0.001	
Serum triacylglycerols (mmol/l)	0.35±0.02	0.52±0.03	0.48±0.01	1.11±0.18	0.57±0.07	0.64±0.08	<0.001	<0.001	<0.01	
Serum cholesterol (mmol/l)	2.65±0.11	2.82±0.13	2.37±0.10	3.60±0.18	3.69±0.32	3.03±0.11	<0.01	NS	NS	
Serum LDL-cholesterol (mmol/l)	0.78±0.11	0.70±0.09	0.64±0.07	1.62±0.13	1.16±0.13	0.92±0.11	<0.001	<0.05	<0.001	
Serum HDL-cholesterol (mmol/l)	1.53±0.06	1.76±0.14	1.43±0.06	1.56±0.26	1.60±0.11	1.69±0.09	NS	NS	NS	
BUN (mmol/l)	5.55±0.18	4.37±0.25	5.08±0.28	8.75±0.53	8.72±0.34	6.28±0.19	<0.001	NS	<0.001	
Serum creatinine (μ mol/l)	19.66±0.67	17.31±1.95	21.13±2.05	57.21±6.15	66.13±7.13	63.85±5.44	<0.001	NS	NS	
Urine volume (ml/day)	1.25±0.14	2.16±0.12	1.60±0.09	13.77±1.23	13.40±2.89	16.07±1.78	<0.001	NS	NS	
GFR (l/24 h)	0.18±0.01	0.17±0.01	0.21±0.02	0.12±0.01	0.13±0.01	0.18±0.01	<0.05	NS	<0.05	
UAE (μ g/mg creatinine)	0.41±0.04	1.02±0.06 ^a	0.31±0.04 ^b	2.62±0.34	1.49±0.26	1.36±0.12	<0.001	<0.001	<0.001	

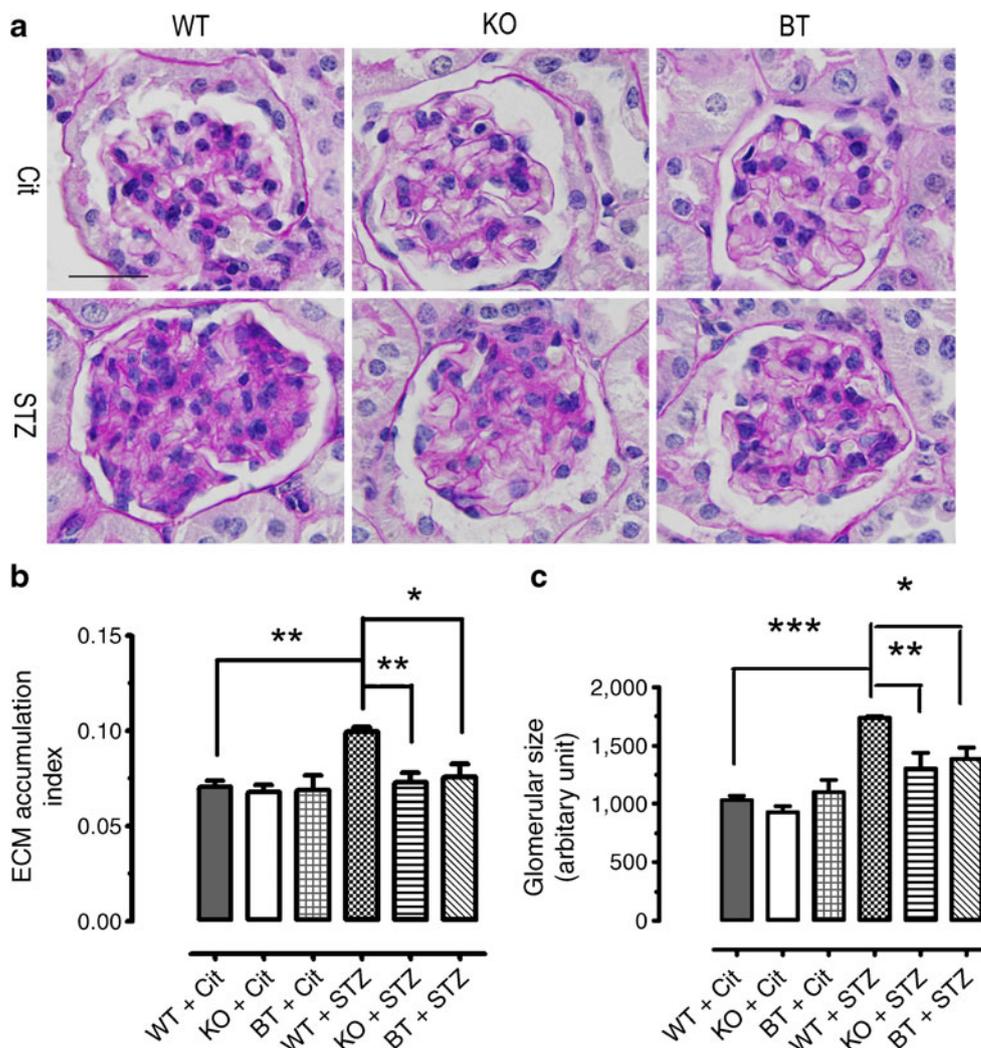
Data are mean \pm SEM, $n=5-6$

The UAE differences among the non-diabetic mice appeared to be consistent with the structural and histomorphological improvement in renal medulla in the BT mice under normal physiological conditions [35]

^a UAE value in the KO + Cit mice is higher than that in the WT + Cit mice but the difference is not statistically significant ($p>0.05$)

^b UAE in the BT + Cit mice is significantly lower than that in the KO + Cit mice ($p<0.05$) but not significantly different from that in the WT + Cit mice ($p>0.05$)

Fig. 1 The effects of genetic deficiency of *Ar* on the pathohistological development of DN in the renal cortex and glomerulus as determined by PAS staining. **a** Renal cortex and glomerulus morphology in six treatment groups of mice at the age of 25 weeks as determined by PAS staining. The results were typical of three mice for each treatment group. Original magnification $\times 1,000$; scale bar, 25 μm . **b** Quantitative analyses of ECM accumulation index in six treatment groups of mice. Values were expressed as the mean \pm SEM, $n=3$; $*p<0.05$, $**p<0.01$. **c** Quantitative analyses of glomerular sizes in six treatment groups of mice. Glomerular area was determined as described in the [Methods](#). Values are expressed as the mean \pm SEM, $n=3$; $*p<0.05$, $**p<0.01$, $***p<0.001$



tissues of the diabetic WT mice in both the intraglomerular cells and extraglomerular cells. In *Ar*-deficient mice, however, renal cortical collagen IV production was significantly reduced in both the *Ar*-KO and BT mice, with the reduction in the glomerular expression being more apparent (Fig. 2a). Western blots of renal cortical tissues further verified the trends of collagen production in diabetic WT, *Ar*-KO and BT mice (Fig. 2b), suggesting that AR deficiency, in glomeruli in particular, led to significant reductions in diabetes-induced overproduction of collagen IV in the glomeruli.

AR deficiency appears to slightly ameliorate diabetes-induced oxidative stress in the renal cortex Recent studies suggest that DN might be closely linked to oxidative stress [39, 40] and that overactivation of AR/polyol pathway contributes significantly to hyperglycaemia-induced oxidative/nitrosative stress [41, 42]. We therefore performed assays to determine glutathione (GSH) and malondialdehyde (MDA) content and the superoxide dismutase (SOD) activity for renal cortical tissues ($n=3$ per group) in the six

study groups of mice (ESM Fig. 3). When the data for all six treatment groups of mice were analysed with one-way ANOVA, no significant difference in GSH or MDA content or SOD activity between genotype groups was observed for either non-diabetic or diabetic mice, which is probably largely because of the limited number of mice. Despite this, pair-wise comparisons showed that the renal cortical GSH was depleted by $\sim 68\%$ in WT + STZ mice vs WT + Cit mice ($p<0.05$, Student's *t* test), manifesting as a significant depletion in GSH consistent with hyperglycaemia-induced oxidative stress. Interestingly, GSH appeared to be partially corrected to $\sim 72\%$ and $\sim 65\%$ of the normal level in the diabetic *Ar*-KO and BT groups, respectively, although the results with $n=3$ per group were not statistically significant. An apparent rise in cortical MDA content in the diabetic cortex, also consistent with excess oxidative stress in the diabetic vs non-diabetic cortex, was also seen in WT + STZ mice vs WT + Cit mice, and an apparent correction was seen in the diabetic *Ar*-KO mice but not in the diabetic BT mice. In contrast to GSH and MDA, SOD activity showed no trend towards change in any of the groups. Together

Fig. 2 Collagen IV production in the renal cortex and glomerulus in six treatment groups of mice at the age of 25 weeks. **a** Collagen IV production in renal cortex and glomerulus in six treatment groups of mice as determined by immunohistochemistry. Original magnification $\times 1,000$; scale bar, 50 μm . The results were typical of three mice for each treatment group. **b** Collagen IV production in renal cortex in six treatment groups of mice as determined by western blots. Each lane represents an independent sample. Protein loading was calibrated using β -actin

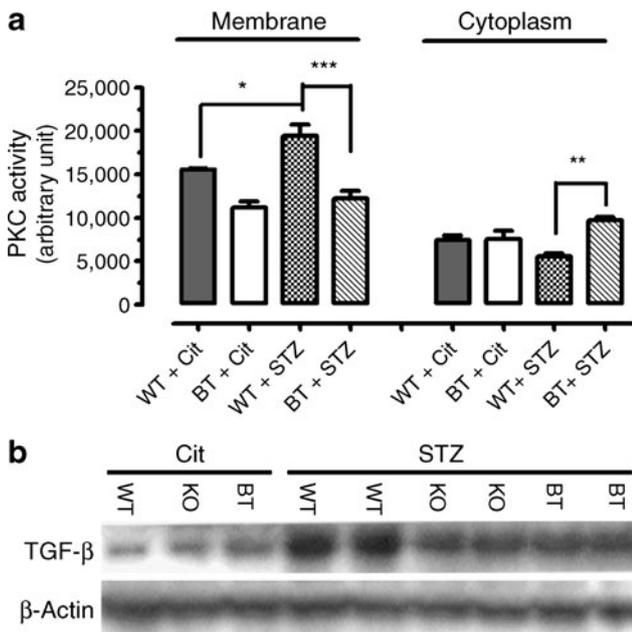
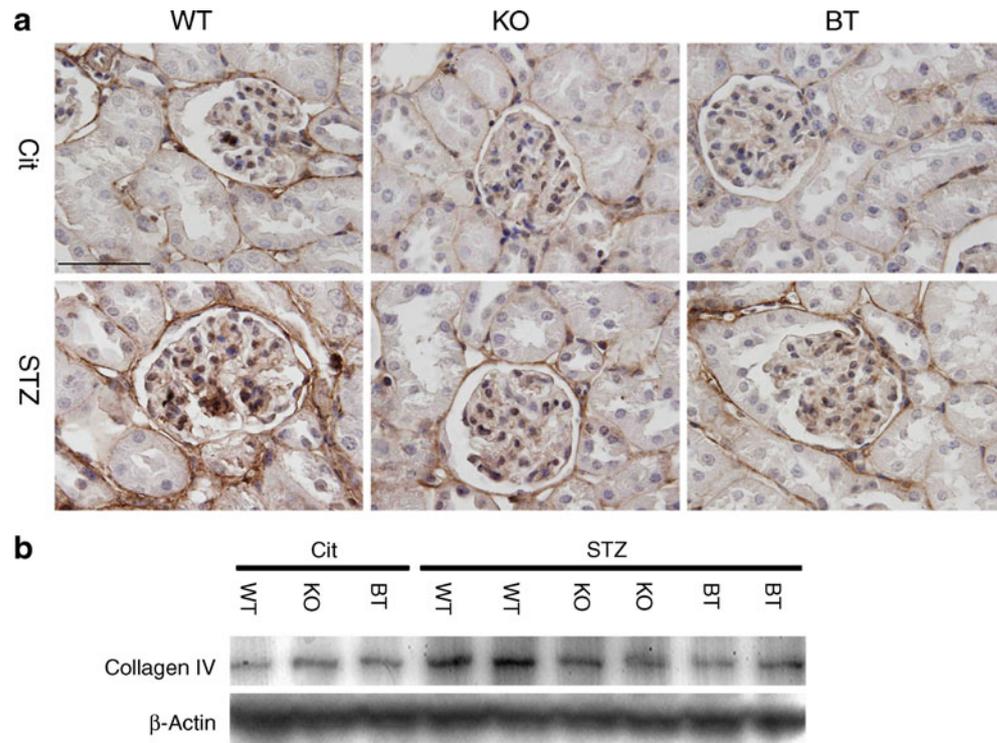


Fig. 3 Effects of *Ar* genetic deficiency on PKC activity and the production of TGF- $\beta 1$. **a** Membrane and cytosolic PKC activities in the renal cortex of four treatment groups of mice at the age of 25 weeks. Values were expressed as the mean \pm SEM, $n=3$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **b** TGF- $\beta 1$ production in the renal cortex in six treatment groups of mice at the age of 25 weeks as determined by western blots. Each lane represents an independent sample. Protein loading was calibrated using β -actin

these data are, in general, consistent with the presence of oxidative stress in the renal cortex of the diabetic vs the non-diabetic state, with a corrective effect of AR deficiency on hyperglycaemia-induced oxidative stress.

AR deficiency in the diabetic KO and BT mice caused significant alterations in PKC activity and TGF- $\beta 1$ production in the renal cortex. Previous studies have established that the overactivation of PKC and TGF- $\beta 1$ plays an important role in the development of DN. Among other roles, the PKC-TGF- $\beta 1$ axis is involved in renal fibrogenesis by regulating the production of a number of components for mesangial expansion. To find out how the genetic deficiency of *Ar* might affect PKC, we determined PKC activity in the cytosolic and membrane fractions of renal cortical homogenates from four treatment groups of mice (i.e. non-diabetic WT and BT mice and diabetic WT and BT mice). As shown in Fig. 3a, 17 weeks of diabetes caused a 24.8% elevation in the renal cortical membrane PKC activity in the BT mice ($19,446 \pm 1,311$ for diabetic WT mice vs $15,572 \pm 144$ for non-diabetic WT mice, $p<0.05$), whereas the cytosolic PKC activity was reduced by about 25%, although this was not statistically significant. In *Ar*-deficient BT mice, however, the diabetes-induced elevation of membrane PKC and downregulation of cytosolic PKC were largely reversed. For the membrane PKC, *Ar* deficiency caused a 37% reduction ($19,446 \pm 1,311$ for diabetic WT mice vs $12,189 \pm 918$ for diabetic BT mice, $p<0.001$). For cytosolic PKC, *Ar* deficiency caused a 75%

increase ($5,534 \pm 366$ for diabetic WT mice vs $9,685 \pm 341$ for diabetic BT mice, $p < 0.01$). Together these results indicate that genetic *Ar* deficiency significantly affected renal cortical PKC activity.

We further performed western blots to analyse renal cortical TGF- β 1 production in non-diabetic and diabetic mice. As shown in Fig. 3b, moderate levels of TGF- β 1 protein were detected in non-diabetic WT, KO and BT mice. Seventeen weeks of diabetes, however, greatly upregulated renal cortical TGF- β 1 production in the diabetic WT mice. In contrast, in both the diabetic *Ar*-KO and BT mice, TGF- β 1 production was significantly reduced and the reduction of TGF- β 1 production is consistent with reduced collagen IV expression in the AR-deficient mice. These data thus suggest that *Ar* deficiency contributes significantly to the amelioration in the development of DN in the diabetic *Ar*-KO and BT mice in part through suppressing diabetes-induced activation of TGF- β 1.

Discussion

As mentioned earlier, a large body of animal and clinical studies using chemically synthesised ARIs has yielded mixed results. As the use of genetic knockout models might avoid the potential problems associated with the incomplete AR inhibition, non-specific inhibition or other side effects of ARIs, these models are crucial for the more definitive elucidation of the role of AR in DN and of the potential utility of AR inhibition in the prevention or treatment of DN. Consistent with the positive findings of studies using ARIs [13, 24, 25, 43–46], we showed in our current study that the genetic deficiency of *Ar* in both the diabetic KO mice and the diabetic BT mice resulted in significantly reduced UAE, mesangial matrix expansion and reduced overexpression of collagen IV, and improved glomerular hypertrophy. Further, consistent with previous observations [10, 44], we demonstrated that *Ar* deficiency was associated with significant attenuation in renal cortical PKC activity and TGF- β 1 production. In addition, *Ar* deficiency significantly ameliorated diabetes-induced renal hypertrophy and elevations in serum triacylglycerols and serum LDL-cholesterol. Together these data clearly imply that hyperglycaemia-induced overactivation of AR and elevation in metabolic flux through the polyol pathway in the renal cortex and the glomeruli contributes significantly to the development of early DN and that inhibition of AR or blockade of the polyol pathway in the renal cortex or the glomeruli might be an effective approach for the prevention or treatment of DN.

Although *Ar* deficiency resulted in ameliorations in a number of important variables associated with DN in both

the diabetic *Ar*-KO and diabetic BT mice, there were a few variables (including serum glucose, BUN and GFR) that characteristically distinguished the diabetic BT mice from the diabetic *Ar*-KO mice. Somewhat surprisingly, the level of BUN in diabetic *Ar*-KO mice at the age of 25 weeks was indistinguishable from that of the diabetic WT mice. In diabetic BT mice, however, BUN was 77% normalised (6.28 ± 0.19 mmol/l for diabetic BT vs 8.75 ± 0.53 mmol/l for diabetic WT, $p < 0.001$), suggesting much improved glomerular function for the diabetic BT mice. The reasons for these differences between the diabetic *Ar*-KO mice and the diabetic BT mice are not clear but are probably linked to the corrections in *Ar*-deficiency-induced renal medullary defects brought about by the re-introduction of renal collecting tubule epithelial-specific production of AR.

PKC signalling has been shown to play critical regulatory roles in the development of DN in experimental models [8]. Because excessive metabolic flux through AR and the polyol pathway is associated with elevation of cytoplasmic NADH/NAD⁺ and diacylglycerol, the latter being a second messenger for PKC, overactivation of AR has been linked with abnormal activation of PKC [9, 11, 47]. Keogh et al. have reported that AR inhibition prevented glucose-induced prostaglandin synthesis and PKC activation [47]. Using the ARI epalrestat, Ishii et al. have also demonstrated that inhibition of AR blocked glucose-induced increases in PKC and TGF- β 1 activity in cultured human mesangial cells [10]. Our demonstration that genetic deficiency of *Ar* led to a decrease in membrane PKC activity but an increase in the cytosolic PKC is consistent with the results from AR inhibition by ARIs. While the mechanisms for the membrane–cytoplasm redistribution of PKC require further investigation, there now remains no doubt that inhibition of AR is a way to suppress glucose-induced overactivation of PKC in the kidneys.

TGF- β 1 is a multifunctional cytokine capable of regulating cell proliferation, differentiation, motility, apoptosis, immune cell function and ECM formation. Currently, this pro-sclerotic protein is recognised as the major cytokine responsible for mesangial expansion, ECM accumulation and vascular dysfunction and glomerulosclerosis in DN [48, 49]. Previous studies have shown that glucose-induced TGF- β 1 is polyol pathway dependent [10, 50] and that inhibition of AR leads to suppressed production of TGF- β [10] and collagen IV [44] and reduced mesangial expansion [45]. The suppression of renal cortical TGF- β , collagen IV and mesangial expansion brought about by ARIs is now further recapitulated with the genetic mouse models deficient in *Ar* in our current investigation. Normalisation of TGF- β 1 activity due to AR deficiency thus is likely to contribute significantly to the amelioration of DN in the *Ar*-KO and BT mice.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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References

- Burg MB, Kador PF (1988) Sorbitol, osmoregulation, and the complications of diabetes. *J Clin Invest* 81:635–640
- Corder CN, Braugher JM, Culp PA (1979) Quantitative histochemistry of the sorbitol pathway in glomeruli and small arteries of human diabetic kidney. *Folia Histochem Cytochem* 17:137–145
- Kasajima H, Yamagishi S, Sugai S, Yagihashi N, Yagihashi S (2001) Enhanced in situ expression of aldose reductase in peripheral nerve and renal glomeruli in diabetic patients. *Virchows Arch* 439:46–54
- Beyer-Mears A, Ku L, Cohen MP (1984) Glomerular polyol accumulation in diabetes and its prevention by oral sorbinil. *Diabetes* 33:604–607
- Kaneko M, Carper D, Nishimura C, Millen J, Bock M, Hohman TC (1990) Induction of aldose reductase expression in rat kidney mesangial cells and Chinese hamster ovary cells under hypertonic conditions. *Exp Cell Res* 188:135–140
- Kikkawa R, Umemura K, Haneda M et al (1992) Identification and characterization of aldose reductase in cultured rat mesangial cells. *Diabetes* 41:1165–1171
- Hohman TC, Carper D, Dasgupta S, Kaneko M (1991) Osmotic stress induces aldose reductase in glomerular endothelial cells. *Adv Exp Med Biol* 284:139–152
- Noh H, King GL (2007) The role of protein kinase C activation in diabetic nephropathy. *Kidney Int Suppl* 106:S49–S53
- Kapor-Drezgic J, Zhou X, Babazono T, Dlugosz JA, Hohman T, Whiteside C (1999) Effect of high glucose on mesangial cell protein kinase C-delta and -epsilon is polyol pathway-dependent. *J Am Soc Nephrol* 10:1193–1203
- Ishii H, Tada H, Isogai S (1998) An aldose reductase inhibitor prevents glucose-induced increase in transforming growth factor-beta and protein kinase C activity in cultured mesangial cells. *Diabetologia* 41:362–364
- Oates PJ, Mylari BL (1999) Aldose reductase inhibitors: therapeutic implications for diabetic complications. *Expert Opin Investig Drugs* 8:2095–2119
- Chung SS, Chung SK (2005) Aldose reductase in diabetic microvascular complications. *Curr Drug Targets* 6:475–486
- Oates PJ (2010) Aldose reductase inhibitors and diabetic kidney disease. *Curr Opin Investig Drugs* 11:402–417
- So WY, Wang Y, Ng MC et al (2008) Aldose reductase genotypes and cardiorenal complications: an 8-year prospective analysis of 1,074 type 2 diabetic patients. *Diab Care* 31:2148–2153
- Heesom AE, Hibberd ML, Millward A, Demaine AG (1997) Polymorphism in the 5'-end of the aldose reductase gene is strongly associated with the development of diabetic nephropathy in type I diabetes. *Diabetes* 46:287–291
- Moczulski DK, Burak W, Doria A et al (1999) The role of aldose reductase gene in the susceptibility to diabetic nephropathy in type II (non-insulin-dependent) diabetes mellitus. *Diabetologia* 42:94–97
- Neamat-Allah M, Feeney SA, Savage DA et al (2001) Analysis of the association between diabetic nephropathy and polymorphisms in the aldose reductase gene in type 1 and type 2 diabetes mellitus. *Diabet Med* 18:906–914
- Shah VO, Scavini M, Nikolic J et al (1998) Z-2 microsatellite allele is linked to increased expression of the aldose reductase gene in diabetic nephropathy. *J Clin Endocrinol Metab* 83:2886–2891
- Xu M, Chen X, Yan L, Cheng H, Chen W (2008) Association between (AC)_n dinucleotide repeat polymorphism at the 5'-end of the aldose reductase gene and diabetic nephropathy: a meta-analysis. *J Mol Endocrinol* 40:243–251
- Moczulski DK, Scott L, Antonellis A et al (2000) Aldose reductase gene polymorphisms and susceptibility to diabetic nephropathy in type 1 diabetes mellitus. *Diabet Med* 17:111–118
- Maeda S, Haneda M, Yasuda H et al (1999) Diabetic nephropathy is not associated with the dinucleotide repeat polymorphism upstream of the aldose reductase (ALR2) gene but with erythrocyte aldose reductase content in Japanese subjects with type 2 diabetes. *Diabetes* 48:420–422
- Yamaoka T, Nishimura C, Yamashita K et al (1995) Acute onset of diabetic pathological changes in transgenic mice with human aldose reductase cDNA. *Diabetologia* 38:255–261
- Ng DP, Hardy CL, Burns WC et al (2002) Prevention of diabetes-induced albuminuria in transgenic rats overexpressing human aldose reductase. *Endocrinology* 18:47–56
- Beyer-Mears A, Mistry K, Diecke FP, Cruz E (1996) Zopolrestat prevention of proteinuria, albuminuria and cataractogenesis in diabetes mellitus. *Pharmacology* 52:292–302
- McCaleb ML, McKean ML, Hohman TC, Laver N, Robison WG Jr (1991) Intervention with the aldose reductase inhibitor, tolrestat, in renal and retinal lesions of streptozotocin-diabetic rats. *Diabetologia* 34:695–701
- Tilton RG, Chang K, Pugliese G et al (1989) Prevention of hemodynamic and vascular albumin filtration changes in diabetic rats by aldose reductase inhibitors. *Diabetes* 38:1258–1270
- Daniels BS, Hostetter TH (1989) Aldose reductase inhibition and glomerular abnormalities in diabetic rats. *Diabetes* 38:981–986
- Faiman G, Ganguly P, Mehta A, Thliveris JA (1993) Effect of statil on kidney structure, function and polyol accumulation in diabetes mellitus. *Mol Cell Biochem* 125:27–33
- McAuliffe AV, Brooks BA, Fisher EJ, Molyneaux LM, Yue DK (1998) Administration of ascorbic acid and an aldose reductase inhibitor (tolrestat) in diabetes: effect on urinary albumin excretion. *Nephron* 80:277–284
- Pedersen MM, Christiansen JS, Mogensen CE (1991) Reduction of glomerular hyperfiltration in normoalbuminuric IDDM patients by 6 mo of aldose reductase inhibition. *Diabetes* 40:527–531
- Cao D, Fan ST, Chung SS (1998) Identification and characterization of a novel human aldose reductase-like gene. *J Biol Chem* 273:11429–11435
- Wallner EI, Wada J, Tramonti G, Lin S, Srivastava SK, Kanwar YS (2001) Relevance of aldo-keto reductase family members to the pathobiology of diabetic nephropathy and renal development. *Ren Fail* 23:311–320
- Barski OA, Gabbay KH, Grimshaw CE, Bohren KM (1995) Mechanism of human aldehyde reductase: characterization of the active site pocket. *Biochemistry* 34:11264–11275
- Ho HT, Chung SK, Law JW et al (2000) Aldose reductase-deficient mice develop nephrogenic diabetes insipidus. *Mol Cell Biol* 20:5840–5846

35. Yang JY, Tam WY, Tam S et al (2006) Genetic restoration of aldose reductase to the collecting tubules restores maturation of the urine concentrating mechanism. *Am J Physiol Renal Physiol* 291:F186–F195
36. Aida K, Ikegishi Y, Chen J et al (2000) Disruption of aldose reductase gene (*Akr1b1*) causes defect in urinary concentrating ability and divalent cation homeostasis. *Biochem Biophys Res Commun* 277:281–286
37. Cohen MP, Lautenslager GT, Shearman CW (2001) Increased urinary type IV collagen marks the development of glomerular pathology in diabetic *d/db* mice. *Metabolism* 50:1435–1440
38. Steffes MW, Bilous RW, Sutherland DE, Mauer SM (1992) Cell and matrix components of the glomerular mesangium in type I diabetes. *Diabetes* 41:679–684
39. Forbes JM, Coughlan MT, Cooper ME (2008) Oxidative stress as a major culprit in kidney disease in diabetes. *Diabetes* 57:1446–1454
40. Kashiwara N, Haruna Y, Kondeti VK, Kanwar YS (2010) Oxidative stress in diabetic nephropathy. *Curr Med Chem* 17:4256–4269
41. Obrosova IG (2005) Increased sorbitol pathway activity generates oxidative stress in tissue sites for diabetic complications. *Antioxid Redox Signal* 7:1543–1552
42. Drel VR, Pacher P, Stevens MJ, Obrosova IG (2006) Aldose reductase inhibition counteracts nitrosative stress and poly(ADP-ribose) polymerase activation in diabetic rat kidney and high-glucose-exposed human mesangial cells. *Free Radic Biol Med* 40:1454–1465
43. Cohen MP (1986) Aldose reductase, glomerular metabolism, and diabetic nephropathy. *Metabolism* 35:55–59
44. Dan Q, Wong RL, Yin S, Chung SK, Chung SS, Lam KS (2004) Interaction between the polyol pathway and non-enzymatic glycation on mesangial cell gene expression. *Nephron Exp Nephrol* 98:e89–e99
45. Itagaki I, Shimizu K, Kamanaka Y et al (1994) The effect of an aldose reductase inhibitor (Epalrestat) on diabetic nephropathy in rats. *Diabetes Res Clin Pract* 25:147–154
46. Donnelly SM, Zhou XP, Huang JT, Whiteside CI (1996) Prevention of early glomerulopathy with tolrestat in the streptozotocin-induced diabetic rat. *Biochem Cell Biol* 74:355–362
47. Keogh RJ, Dunlop ME, Larkins RG (1997) Effect of inhibition of aldose reductase on glucose flux, diacylglycerol formation, protein kinase C, and phospholipase A2 activation. *Metabolism* 46:41–47
48. Sharma K, McGowan TA (2000) TGF-beta in diabetic kidney disease: role of novel signaling pathways. *Cytokine Growth Factor Rev* 11:115–123
49. Zhu Y, Usui HK, Sharma K (2007) Regulation of transforming growth factor beta in diabetic nephropathy: implications for treatment. *Semin Nephrol* 27:153–160
50. Wong TY, Phillips AO, Witowski J, Topley N (2003) Glucose-mediated induction of TGF-beta 1 and MCP-1 in mesothelial cells in vitro is osmolality and polyol pathway dependent. *Kidney Int* 63:1404–1416