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Redox state-dependent and sorbitol accumulation-independent diabetic albuminuria in mice with transgene-derived human aldose reductase and sorbitol dehydrogenase deficiency

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Abstract

Aims/hypothesis. We investigated the role played by sorbitol accumulation in the kidney in the development of diabetic albuminuria.
Methods. We created mice (*hAR-Tg:SDH* null) with transgene-derived human aldose reductase and sorbitol dehydrogenase (SDH) deficiency, and analysed (i) the contribution of accumulated sorbitol to urinary albumin excretion rate, and (ii) the effect of the aldose reductase inhibitor, epalrestat, on the diabetic redox state, including decreased renal reduced glutathione concentrations or increased lactate to pyruvate ratios in the diabetic kidney.
Results. Compared to littermates, non-diabetic transgenic mice had a 2.6-fold increase in aldose reductase mRNA. In a diabetic group, aldose reductase mRNA in *hAR-Tg* mice was 2.7-fold higher than in littermates. In the diabetic and non-diabetic groups, *hAR-Tg:SDH* null mice had the highest sorbitol content among all four genetic types including *hAR-Tg:SDH* null, *SDH* null, *hAR-Tg* and littermates. The urinary albumin excretion rate in non-diabetic groups was similar in the four genetic types of mouse. In diabetic groups it was greater than in non-diabetic groups, but did not correlate with the sorbitol content among the four genetic types of mouse. When aldose reductase inhibitor and streptozotocin were given simultaneously at 6 weeks of age, epalrestat prevented diabetic increases in urinary albumin excretion rate and completely prevented diabetic decreases in reduced glutathione concentrations and diabetic increases in lactate to pyruvate ratios, even in the presence of transgenic aldose reductase.
Conclusions/interpretation. The degree of diabetic albuminuria in genetically modified mice is dependent on the redox state and independent of polyol accumulation; aldose reductase inhibitor can prevent diabetic albuminuria by normalising diabetic redox changes. [Diabetologia (2004) 47:541–548]

Keywords Transgenic mice · Aldose reductase · Sorbitol dehydrogenase · Diabetic · Albuminuria · Redox change

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Abbreviations: AR, Aldose reductase · SDH, sorbitol dehydrogenase · UAE, urinary albumin excretion rate · GSH, reduced glutathione · hAR-Tg, human aldose reductase-transgenic mouse · L/P, lactate/pyruvate

Diabetic complications are the major cause of morbidity and mortality in patients with diabetes. Although hyperglycaemia is an important determinant in the pathogenesis of diabetic microvascular complications, the mechanisms by which this happens remain controversial [1, 2, 3]. As the prevalence of diabetic nephropathy is increasing, it is urgently necessary to identify its pathophysiological mechanisms. Four major pathways, including increased glucose flux through the polyol pathway, activation of protein kinase C, increased formation of AGE, and increased hexosamine pathway flux, have been proposed for diabetic nephropathy [1, 2, 3]. Many studies have shown that diabe-

tes and hyperglycaemia increase oxidative stress, but it is unknown whether oxidative stress is localised upstream or downstream of the four major pathways [1, 2]. A recent study showed that normalisation of increased mitochondrial oxidant production prevented hyperglycaemia-induced activation of three pathways, including the polyol pathway, the protein kinase C pathway and formation of AGE [4]. However, the role of oxidative stress in increasing the polyol pathway has not been confirmed in other diabetic animal models, where the polyol pathway was not suppressed by antioxidant [5].

Previous studies on regulation of the polyol pathway in the kidney depended on aldose reductase (AR) inhibitors as a tool, yet inhibition of the polyol pathway in vivo by AR inhibitor has yielded inconsistent results. Some investigators have reported that AR inhibitor has a beneficial effect on the development of albuminuria [6, 7, 8], whereas others found no beneficial effects [9, 10, 11]. The polyol pathway converts glucose to sorbitol by NADPH-dependent AR (E.C. 1.1.1.21), and sorbitol to fructose by NAD^+-dependent sorbitol dehydrogenase (SDH) (E.C. 1.1.1.14). Increased flux of the polyol pathway causes metabolic changes including: (i) sorbitol accumulation; (ii) a decreased amount of available NADPH due to AR reaction; (iii) pseudohypoxia with increased NADH/NAD^+ or increased lactate to pyruvate (L/P) ratios mediated by SDH; and (iv) activation of protein kinase C by increasing de novo diacylglycerol synthesis [1, 2, 3].

Although the absence of AR produces viable mice with nephrogenic diabetes insipidus [12], the role of polyol accumulation and/or associated redox changes for diabetic albuminuria remains inconclusive. To study the contribution of renal sorbitol accumulation and AR inhibitor to urinary albumin excretion rate (UAE) and redox changes in the diabetic kidney, we made four genetically modified mice. First a human aldose reductase-transgenic mouse (*hAR-Tg*) in which *hAR* gene was expressed under the murine *MHC* class *I* promoter was generated [13, 14, 15]. To achieve higher polyol accumulation in the kidney, we cross-bred *hAR-Tg* with homozygous SDH-deficient mice (C57BL/LiA; *SDH* null) [16] to obtain *hAR-Tg:SDH* null mice. We then analysed the contribution of accumulated sorbitol to UAE and the effects of AR inhibitor on diabetic redox state reflected by a decrease in renal reduced glutathione (GSH) concentrations or increased L/P ratios in the diabetic kidney.

Materials and methods

Detection of hAR-Tg. We generated the *hAR-Tg* by using a transgene consisting of the murine *H-2K*^{d} promoter fused to a human *AR* coding sequence [13]. The BDF1 founder mouse was bred with BDF1, and integration of the transgene was detected by PCR, as described [14].

Detection of SDH null. Genomic DNA was extracted from punched ear lobes at 4 weeks of age. To amplify the region of exon 8 and intron 8 of the mouse *SDH* gene, oligonucleotide primers were designed [17, 18]. The experiment used a sense-primer in exon 8 (5′-ACTCACTCTGGT GGGACCT-3′), and an antisense-primer in intron 8 (5′-ATCGGCCACCTGTAA-GAGAT-3′). An amplified fragment of 0.8 kb in size was digested with *Pma*CI. The undigested PCR product due to the point mutation of G to A at +1 splicing donor site in intron 8 showed the presence of the *SDH*-deficient allele.

Cross-breeding of hAR-Tg with SDH null mice. We cross-bred *hAR-Tg* in BDF1 genetic background with *SDH* null in C57BL genetic background, and their offspring (F_1) were then intercrossed with each other. We then generated F_2 progenies of *hAR-Tg:SDH* null, which were hemizygous for *hAR-Tg* and homozygous for *SDH*-deficient alleles. *SDH* null mice were provided by Dr. R.G.M. ten Berg of the Netherlands Cancer Institute and Prof. S.S.M. Chung of the Institute of Molecular Biology, University of Hong Kong.

Grouping of experimental animals. Mice with four genetic types including *hAR-Tg:SDH* null, *SDH* null, *hAR-Tg*, and littermates were assigned to three groups at 6 weeks of age. Group 1 (control) included non-diabetic mice fed with standard chow (Oriental Bio-Service, Kyoto, Japan). Group 2 (diabetes) included mice made diabetic by a single intraperitoneal dose of streptozotocin (Wako Pure Chemicals, Osaka, Japan; 200 mg/kg male body weight, 220 mg/kg female body weight) in 50 mmol/l citrate buffer, pH 4.5, at 6 weeks of age. Mice with non-fasting blood glucose concentrations higher than 14 mmol/l were considered to be diabetic. Group 3 (diabetes + AR inhibitor) included diabetic mice treated with AR inhibitor. The AR inhibitor epalrestat (5-[(1*Z*, 2*E*)-2-methyl-3-phenylpropenylidene]-4-oxo-2-thioxo-3-thiazolidine acetic acid) (Ono Pharmaceutical, Osaka, Japan) was given to Group 3 mice in standard chow at a final concentration of 0.08% (w/w), starting on the day streptozotocin was given at 6 weeks of age.

Glycaemic control and mean body weight between Groups 2 and 3 were not statistically different. The number of mice in each group ranged from four to eight, and an equal number of male or female mice was used. The mice were kept on a light and dark cycle of 12 h each, alternating at 08.00 and 20.00 hours, and had free access to food. Care and treatment of the animals was in compliance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). The protocol was approved by the University of Tokushima institutional review board for animal procedures.

Northern blot analysis of AR mRNA. To detect expression levels of *AR* mRNA including endogenous mouse *AR* mRNA and transgene-derived *hAR* mRNA, total RNA was extracted from snap-frozen kidneys at 6, 7 and 10 weeks of age using the acid guanidium thiocyanate-phenol-chloroform method [19]. Total RNA (10 µg) was applied to 1% agarose gel and transferred to a nylon membrane (Gene Screen Plus, Dupont, Boston, Mass., USA). The membrane was hybridised with ^{32}P-labelled DNA probes. Of these, three were different *AR*-specific probes and one was a ribosomal RNA-specific probe. The nucleotide numbers of probes were as follows: (i) 969–1312 of the 3′-portion of mouse *AR* cDNA [20] for mouse *AR* mRNA detection; (ii) 994–1284 of the 3′-portion of human *AR* cDNA [21] for human *AR* mRNA detection; (iii) 112–1284 of human *AR* cDNA sequence for the total amount of *AR* mRNA detection; and (iv) 29 antisense nucleotide sequences against 3690–3718 of the mouse 28S ribosomal RNA as an internal control [22]. Because northern blot analysis was unable to differentiate mouse

AR mRNA and human *AR* mRNA in size, the 3′-portion of mouse *AR* or human *AR* cDNA probes was used. The 3′-probes did not cross-hybridise each other in stringent conditions. The coding probe of human *AR* cDNA with the 86% nucleotide identity of mouse *AR* cDNA was hybridised to both human and mouse *AR* mRNA. We semi-quantitated *AR* mRNA levels with Fujix-BAS (Fuji-Film, Tokyo, Japan) after standardisation for the 28S ribosomal RNA signal.

Immunohistochemical detection of hAR and measurement of hAR in the kidney. Localisation of transgene-derived hAR in the kidney was examined by immunohistochemistry with the avidin-biotin peroxidase complex kit (Vector Labs, Burlingame, Calif., USA) [23] and a rabbit polyclonal antibody against hAR [13]. The hAR contents in the kidneys of Group 1 (control) and Group 2 (diabetes) were assayed at 6, 7 and 10 weeks of age with ELISA [24].

Measurement of renal sorbitol content. Sorbitol was assayed with GC-MS. Snap-frozen kidneys were homogenised with 4 volumes of wet tissue weight of water containing U-[^{13}C]-sorbitol as an internal standard. After precipitating protein with 9 volumes of ethanol, the supernatant was lyophilised. The lyophilised sample was heated at 90°C for 40 min with 100 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (Wako) and 10 µl of chlorotrimethylsilane (Tokyo Kasei Kogyo, Tokyo, Japan). We then analysed 2 µl using a QP-5050A gas chromatograph (Shimadzu, Kyoto, Japan) with an Ultra Alloy 5 silica capillary column (Frontier Lab, Kohriyama, Japan) and a split injector (ratio 20:1). Helium with a flow-rate of 1.47 ml/min was used as carrier gas. The column temperature was programmed to rise from 100 to 220°C at a rate of 40°C per min and from 220 to 325°C at a rate of 20°C per min.

Assessment of UAE. At 6, 7 and 10 weeks of age, UAE was assessed from the mean of three consecutive 24-h urine collections from each mouse using a metabolic cage (Natsume, Tokyo, Japan). Urine albumin concentrations were assayed using an Albuwell M kit (Exocell, Philadelphia, Pa., USA).

Measurement of GSH concentrations and L/P ratios. We calculated GSH concentrations by subtracting the oxidised glutathione concentration from the entire glutathione concentration. Kidneys were homogenised in 1% 5-sulfosalicylic acid, and the homogenates were centrifuged. Glutathione in the clear supernatant was assayed by the enzymatic recycling procedure, sequentially oxidised by 5,5′-dithiobis (2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase. The rate of 2-nitro-5-thiobenzoic acid formation was monitored at 412 nm [25]. Oxidised glutathione concentrations were assayed after 2-vinyl-pyridine conjugation of GSH [25].

To assay L/P ratios, kidneys were homogenised in 0.12 mol/l perchloric acid and centrifuged. The clear supernatant was neutralised by KOH [26, 27]. Concentrations of lactate and pyruvate were measured with lactate oxidase and pyruvate oxidase respectively. Reaction buffers of DETERMINER LA (Kyowa Hakko, Tokyo, Japan) and DETERMINER PA were used to assay lactate and pyruvate respectively. The lactate or pyruvate content in the kidney was calculated by subtracting the amount of lactate or pyruvate in the blood. The fraction of the blood was determined by the proportion of oxyhaemoglobin [26, 27].

Statistical analysis. Results were expressed as means ± SEM. Comparisons between individual groups at the designated time points were made with Student's unpaired *t* test. A *p* value of less than 0.05 was considered statistically significant.

Results

Northern blot analysis and detection of human AR with ELISA. Northern blot analysis showed that the total amount of *AR* mRNA including mouse and human *AR* in non-diabetic *hAR-Tg* was 2.6-fold higher than in littermates at 6 weeks of age (Fig. 1). In Group 2 (diabetes), the total amount of *AR* mRNA in *hAR-Tg* at 10 weeks of age was 2.7-fold higher than that in littermates (Fig. 1). The amount of hAR protein assayed with ELISA in the kidney of *hAR-Tg* was 393±31 µg/g protein. In *hAR-Tg* in Group 2 (diabetes) hAR protein concentrations at 7 and 10 weeks of age were 2.8- and 2.4-fold higher than in Group 1 (control) respectively ($p<0.0001$ and 0.0005).

Immunohistochemical detection of hAR. Immunohistochemical study in *hAR-Tg* mice showed that hAR was expressed not only in the medulla, but also in the glomerular mesangial cells and the epithelial cells of distal tubules of the kidney (Fig. 2a). No hAR was detected in littermates (Fig. 2b).

Renal sorbitol content. The renal sorbitol content at 6 weeks of age was 276±9 µg/g wet weight in *hAR-Tg:SDH* null, 177±18 µg/g wet weight in *SDH* null, 133±24 µg/g wet weight in *hAR-Tg*, and 125±7 µg/g wet weight in littermates. In control groups at both 6 and 10 weeks of age, *hAR-Tg:SDH* null had the highest renal sorbitol content of all four genetic types ($p<0.05$) (Fig. 3a-d). Renal sorbitol contents in the

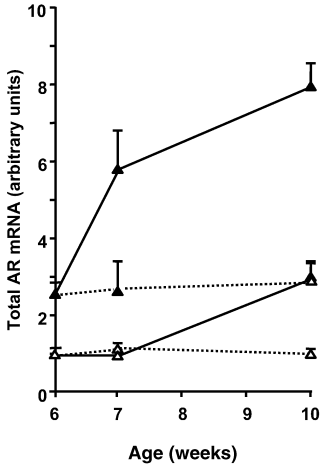

Fig. 1. Changes in aldose reductase mRNAs. Levels of mRNA were semi-quantitated with Fujix-BAS after standardisation for the signal for 28S ribosomal RNA. Changes in total amount of mRNA, including mouse and human aldose reductase (*AR*) mRNA detected with the human *AR* coding probe, are shown. The standardised signal intensity for mouse *AR* mRNA was taken as one arbitrary unit. Group 1 (control) included control littermates (△····) and control human aldose reductase-transgenic mice (*hAR-Tg*) (▲····); Group 2 (diabetes) included diabetic littermates (△—) and diabetic *hAR-Tg* (▲—). Data in means ± SE are plotted against the age of mice in weeks

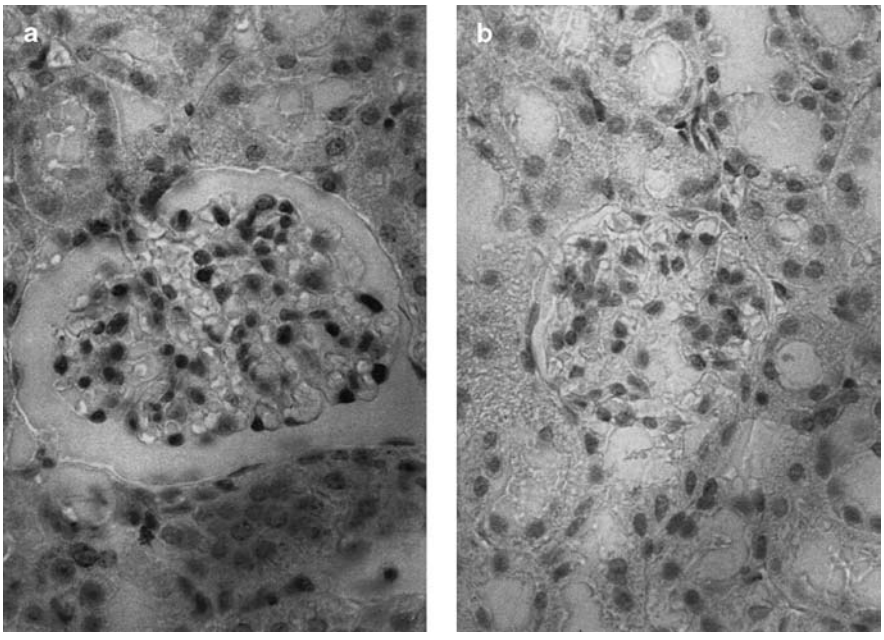

Fig. 2a, b. Immunohistochemical detection of human aldose reductase (*hAR*) in the kidney was done in the kidney of human aldose reductase-transgenic mice (*hAR-Tg*) (**a**), but not in littermates (**b**)

control group did not change from 6 to 10 weeks (Figs. 3a–d). In diabetic groups, renal sorbitol content in *hAR-Tg:SDH* null was the highest at 7 weeks of age, and was higher than in *hAR-Tg* and littermates at 10 weeks ($p<0.05$) (Figs. 3a–d). In diabetic mice at 10 weeks of age, treatment with epalrestat reduced renal sorbitol contents to 78%, 88%, 65% and 89% of the corresponding diabetic groups in *hAR-Tg:SDH* null, *SDH* null, *hAR-Tg* and littermates respectively (Figs. 3a–d).

Assessment of UAE. In diabetic mice, UAE in all four genetic types was much higher than in the control groups (Fig. 4a–d). However, UAE levels were not different between the four genetic types of diabetic mice. Epalrestat decreased UAE in all four genetic types of diabetic mice, but not completely to the control levels (Fig. 4a–d).

Correlation between UAE and renal sorbitol content. No correlations between UAE and renal sorbitol content were seen, either in both non-diabetic or in diabetic groups (Fig. 5).

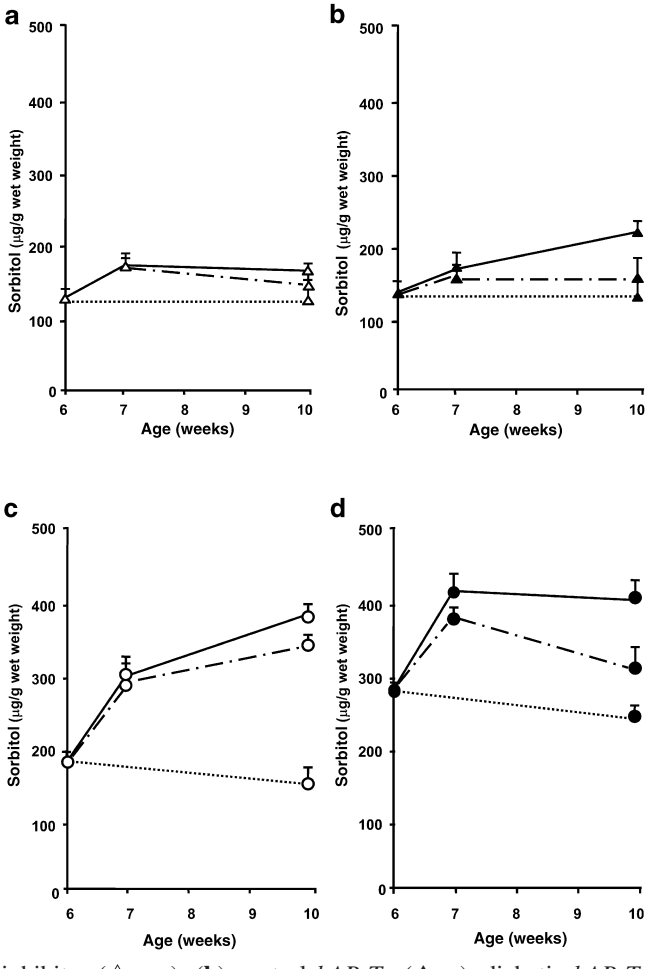

Fig. 3a–d. Changes in renal sorbitol contents. Sorbitol contents as μg/g kidney wet weight are shown for littermates (**a**), human aldose reductase-transgenic mice (*hAR-Tg*) (**b**), sorbitol dehydrogenase (*SDH*) null (**c**) and *hAR-Tg:SDH* null (**d**). Symbols stand for: (**a**) control littermates (△····), diabetic littermates (△—), diabetic littermates treated with aldose reductase (AR) inhibitor (△- · -); (**b**) control *hAR-Tg* (▲····), diabetic *hAR-Tg* (▲—), diabetic *hAR-Tg* treated with AR inhibitor (▲- · -); (**c**) control *SDH* null (○····), diabetic *SDH null* (○—), diabetic *SDH null* treated with AR inhibitor (○- · -); and (**d**) control *hAR-Tg:SDH null* (●····), diabetic *hAR-Tg:SDH null* (●—), and diabetic *hAR-Tg:SDH* null treated with AR inhibitor (●- · -)

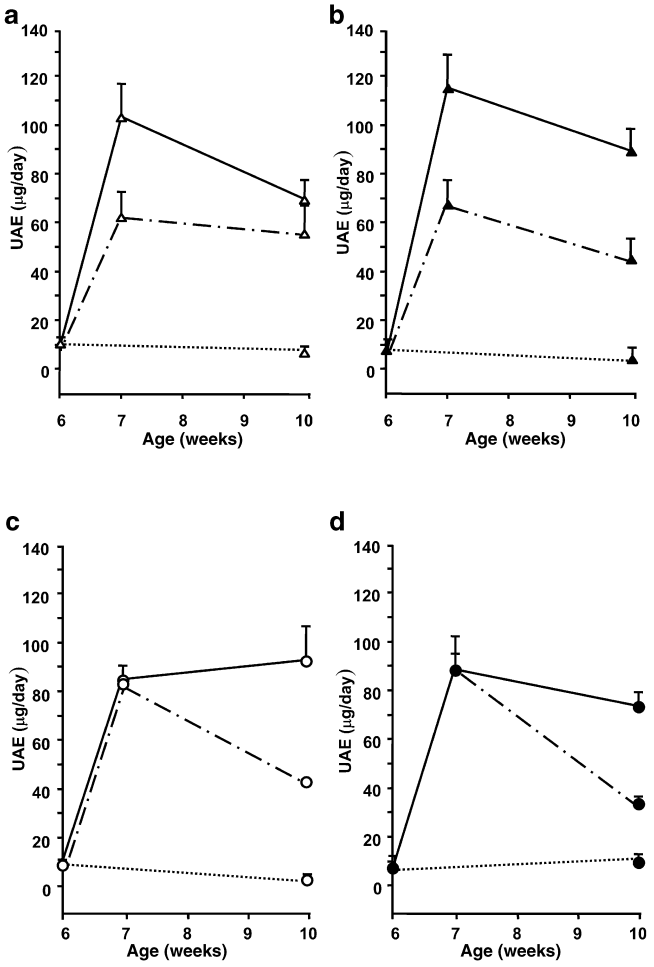

Fig. 4a–d. Changes in urinary albumin excretion rates (UAE). Changes in μg/day are shown for littermates (**a**), human aldose reductase-transgenic mice (*hAR-Tg*) (**b**), sorbitol dehydrogenase (*SDH*) null (**c**), and *hAR-Tg:SDH* null (**d**). Symbols stand for: (**a**) control littermates (△····), diabetic littermates (△—), diabetic littermates treated with aldose reductase (AR) inhibitor (△- · -); (**b**) control *hAR-Tg* (▲····), diabetic *hAR-Tg* (▲—), diabetic *hAR-Tg* treated with AR inhibitor (▲- · -); (**c**) control *SDH* null (○····), diabetic *SDH* null (○—), diabetic *SDH* null treated with AR inhibitor (○- · -); and (**d**) control *hAR-Tg:SDH* null (●····), diabetic *hAR-Tg:SDH* null (●—), and diabetic *hAR-Tg:SDH* null treated with AR inhibitor (●- · -)

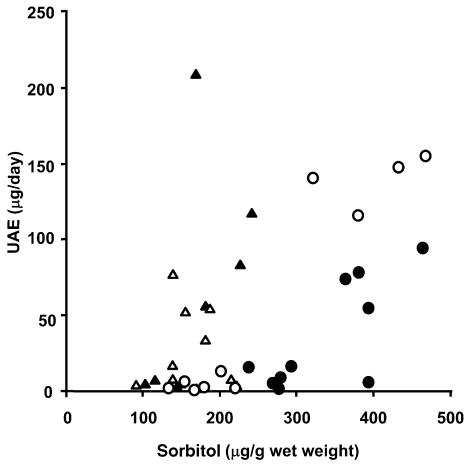

Fig. 5. Absence of correlation between urinary albumin excretion rate (UAE) and sorbitol contents at 10 weeks of age. Symbols stand for: littermates (△), human aldose reductase-transgenic mice (*hAR-Tg*) (▲), sorbitol dehydrogenase (*SDH*) null (○), and *hAR-Tg:SDH* null (●). No correlations were observed

GSH concentrations and L/P ratios. Concentrations of GSH at 6 weeks of age were 3.30±0.09 (n=4) and 3.38±0.07 (n=4) μmol/g wet weight in *hAR-Tg* and littermates respectively. In *hAR-Tg* and littermates, GSH concentrations in diabetic groups were lower than in non-diabetic groups at 7 and 10 weeks of age (Figs. 6a, b). Treatment with AR inhibitor brought GSH concentrations to normal, non-diabetic levels in diabetic *hAR-Tg* and littermates (p<0.05, Fig. 6a, b).

Levels of lactate and pyruvate in the kidneys of *hAR-Tg* and littermates are shown in Table 1. L/P ratios were assayed as a surrogate marker of renal cytosolic NADH/NAD$^+$ ratios. In littermates at 10 weeks of age, the L/P ratio in diabetic groups was 3.1-fold higher than in non-diabetic groups (Fig. 6c). In *hAR-Tg* at 10 weeks of age, the L/P ratio in a diabetic

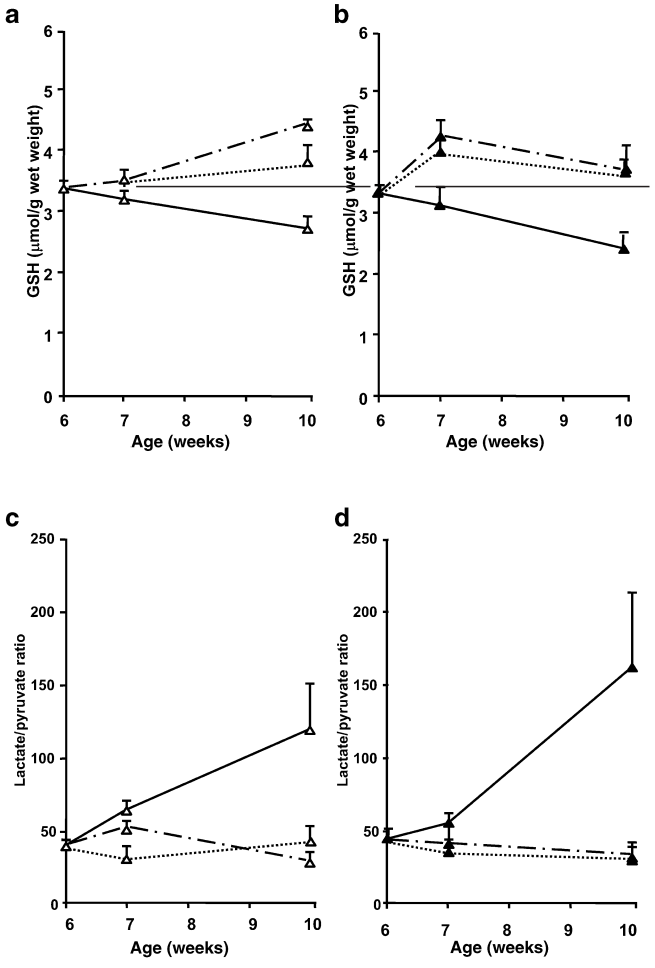

Fig. 6a–d. Changes in reduced glutathione levels and lactate to pyruvate ratios. Changes in reduced glutathione (GSH) levels are shown for littermates (**a**) and aldose reductase-transgenic mice (*hAR-Tg*) (**b**). Lactate to pyruvate (L/P) ratios are shown for littermates (**c**) and *hAR-Tg* (**d**). Symbols stand for: (**a, c**) control littermates (△····), diabetic littermates (△—), diabetic littermates treated with aldose reductase (AR) inhibitor (△- · -); and (**b, d**) control *hAR-Tg* (▲····), diabetic *hAR-Tg* (▲—), and diabetic *hAR-Tg* treated with AR inhibitor (▲- · -)

Table 1. Concentrations of lactate and pyruvate in kidneys of littermates and human aldose reductase-transgenic mice

	Littermates		hAR-Tg	
	Lactate	Pyruvate	Lactate	Pyruvate
Control				
6 weeks	266.8±32.7	6.9±0.8	212.4±31.0	4.9±0.7
7 weeks	211.6±46.7	8.3±2.6	264.3±52.1	7.5±0.4
10 weeks	175.6±32.0	4.9±0.8	146.3±18.9	7.7±1.3
Diabetes				
7 weeks	278.5±36.8	4.9±0.9	276.4±23.6	5.2±0.9
10 weeks	293.4±17.8	3.1±0.5	314.8±47.0	2.8±0.8
Diabetes + ARI				
7 weeks	285.1±35.8	5.3±0.6	275.3±33.2	5.4±1.1
10 weeks	192.7±32.4	6.7±1.2	199.7±32.4	6.0±1.7

Values are in µg/g wet weight and are means ± SE, n=5 to 9. *hAR-Tg*, human aldose reductase-transgenic mice

group was 5.3-fold higher than in a non-diabetic group (Fig. 6d). Treatment with AR inhibitor brought L/P ratios to normal, non-diabetic levels in *hAR-Tg* and littermates of diabetic groups (p<0.05, Fig. 6c, d).

Discussion

Several hyperglycaemia-linked mechanisms singly or in concert contribute to the development of diabetic complications [1, 2]. Excessive accumulation of polyol has been postulated as a factor for diabetic nephropathy. Animal studies in rats have been conducted on the role of polyol pathway for the development of diabetic nephropathy [6, 8, 9, 10]. In this study, we created four different genetically modified mice to attain the maximum accumulation of renal sorbitol by cross-breeding *hAR-Tg* and SDH-deficient mice.

The *hAR-Tg* which we established was a suitable mouse model to study the pathogenesis of diabetic neuropathy [28]. Diabetic *hAR-Tg* had decreased motor nerve conduction velocity and increased myelinated fibre atrophy, which were prevented by giving AR inhibitor. Moreover, the increase of *AR* mRNA observed by us in diabetic mice was consistent with other reports showing 1.8- to 3.5-fold increases in streptozotocin-induced diabetic rats [29, 30]. In the diabetic group, total *AR* mRNA in *hAR-Tg* was 2.7-fold higher than in littermates. In this condition, the sorbitol content was only 1.7-fold higher than in littermates, presumably because sorbitol is rapidly metabolised by SDH.

The *SDH* null mouse (C57BL/LiA mouse) has a single base mutation from G to A at the +1 position of the splicing donor site of intron 8 in the *SDH* gene, which leads to SDH deficiency [18]. We created progeny of *hAR-Tg:SDH* null, in which *SDH*-deficient alleles were homozygously present in *hAR-Tg*. In a non-diabetic condition, the renal sorbitol content in *hAR-Tg:SDH* null was much higher than in mice with the three other genotypes including *SDH* null, *hAR-Tg* and littermates. Renal sorbitol content in diabetic *hAR-Tg:SDH* null was comparable to that in diabetic *SDH* null, but was higher than in diabetic *hAR-Tg* and littermates. Although UAE is a good marker of diabetic nephropathy, no correlation was observed between renal sorbitol content and UAE. The possibility that minor differences (at most 3.5-fold) in sorbitol content in the diabetic group of four genotypes mask a positive correlation between sorbitol content and UAE cannot be completely denied. Greater accumulation of sorbitol in the lens due to SDH deficiency and transgenic *hAR* expression by the *crystallin* promoter has been shown to lead to the formation of cataracts [31]. However, motor nerve conduction velocity in the diabetic condition was not affected by nerve sorbitol content in *SDH* null mice [32]. Our results strongly suggest that polyol accumulation in the kidney is not a main cause of diabetic albuminuria in our mouse models.

Oxidative stress is widely recognised as a key component in the development of diabetic complications [33], but the relation between oxidative stress and polyol pathway is controversial. Both AR inhibitors and antioxidants are effective in preventing or delaying diabetic complications [2]. In contrast, some studies suggest that AR, which metabolises the toxic products of lipid peroxidation, protects humans from diabetic complications [34]. In contrast to the anti-oxidative effect of AR, another study reported that transgenic *hAR* expression by the *crystallin* promoter exacerbated diabetes-induced lipid peroxidation and GSH depletion in the lens [35]. In addition, a decrease in lipid aldehyde accumulation by structurally different AR inhibitors was observed [5, 36, 37]. These results suggest that inhibition of AR activity is more likely to result in the protection of tissue from oxidative injury in diabetic complications.

According to the concept of diabetic pseudohypoxia [3], an increase in glucose flux through the polyol pathway and increase in the NADH to NAD^+ ratio due to SDH-dependent oxidation of sorbitol to fructose in diabetes constitute a mechanism of diabetic complications. This SDH-dependent pseudohypoxia is maintained in equilibrium by a coupled increase in the L/P ratio, which serves as a surrogate marker for the cytosolic ratio of free NADH/NAD^+ [3]. Recent studies on diabetic neuropathy and cataract using SDH inhibitors [38, 39, 40] and an SDH-deficient mouse model [32] excluded diabetic pseudohypoxia or SDH as the reason for diabetic complications. In addition, our finding that SDH-deficiency did not improve the increased UAE in diabetes does not support the pseudohypoxia hypothesis.

Aldose reductase uses NADPH to reduce glucose to sorbitol, which is then oxidised to fructose via SDH

using NAD^+ as a cofactor. The competition between AR and glutathione reductase for the cofactor NADPH in diabetes is the most likely cause for GSH depletion. In diabetic *hAR-Tg* and diabetic littermates, decreases in GSH concentrations and increases in L/P ratios were of a comparable size in *hAR-Tg* and littermates, suggesting that the amount of AR is not rate-limiting for the diabetic redox changes in the kidney. AR inhibitor normalised not only the diabetic decrease in GSH concentrations, but also the diabetic increase in L/P ratios even in the presence of transgenic *hAR*. The increased L/P ratio of diabetic mice in our study is consistent with a report showing an increase in L/P ratios in rat glomerular mesangial cells exposed to glucose [41]. Because SDH-deficiency did not improve the diabetic increase in UAE, our study did not analyse GSH concentrations and L/P ratios in kidneys of *SDH* null or *hAR-Tg:SDH* null mice. In our study, AR inhibitor not only reduced polyol content, but also prevented oxidative stress by normalising redox changes in diabetic kidney. These results strongly suggest that inhibition of AR prevents diabetic complications mainly by preventing oxidative stress.

The cross-breeding of genetically modified mice and the generation of mice with four different genotypes in our study led to a more than threefold increase in sorbitol accumulation. The wide-range accumulation of renal sorbitol and the effects of AR inhibitor first made it possible to differentiate the contribution of sorbitol accumulation and redox changes to diabetic albuminuria. The discordance between renal sorbitol content and diabetic albuminuria in mice with all four genetic types strongly suggests that the accumulation of sorbitol in itself is not sufficient to produce diabetic albuminuria. AR inhibitor improved diabetic increases in UAE at 10 weeks of age and completely normalised multiple diabetic redox changes, including the decrease in renal GSH concentrations and the increase in L/P ratios. In summary, the degree of diabetic albuminuria in mice with modified single gene or double genes of the polyol pathway is dependent at least in part, if not totally, on the redox state and independent of polyol accumulation. AR is apparently localised upstream of diabetic oxidative stress. Based on these findings, we conclude that diabetic albuminuria in *hAR*-transgenic and SDH-deficient mice is dependent on the redox state and independent of sorbitol accumulation. We also conclude that AR inhibitor can partly prevent diabetic albuminuria by normalising diabetic oxidative stress.

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