

Superior renoprotective effects of combination therapy with ACE and AGE inhibition in the diabetic spontaneously hypertensive rat

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Abstract

Aims/hypothesis. Diabetic renal disease has been postulated to progress as a result of an interaction between metabolic and haemodynamic pathways. Our aim was to assess the functional, structural, molecular and cellular aspects of renal disease in an experimental model of diabetes with associated hypertension.

Method. Streptozotocin-induced diabetic spontaneously hypertensive rats were randomised to no treatment, the ACE inhibitor, perindopril (2 mg/l), the AGE formation inhibitor, aminoguanidine (1 g/l) and a combination of both agents and were followed for 32 weeks.

Results. Diabetes was associated with a considerable increase in albumin excretion rate. Both aminoguanidine and perindopril retarded the increase in albuminuria, which was completely abrogated by combination therapy. Glomerulosclerosis and tubulointerstitial damage was reduced by both monotherapies with further renoprotection afforded by combination therapy in

both cases. Combination therapy was also associated with a superior restoration in diabetes-induced nephrin protein depletion compared to either monotherapy. *TGFβ1* expression as assessed by in situ hybridisation was increased in the diabetic rats and reduced by perindopril and aminoguanidine.

Conclusion/interpretation. These findings indicate that in the context of diabetes-related renal injury, blocking both the renin-angiotensin and advanced glycation pathways offers superior renoprotection and could be considered as a therapeutic strategy in the prevention and retardation of progressive-diabetic renal injury. [Diabetologia (2004) 47:89–97]

Keywords Diabetic nephropathy · albuminuria · glomerulosclerosis · tubulointerstitial injury · combination therapy · AGE formation · renin-angiotensin system · aminoguanidine · spontaneously hypertensive rat · transforming growth factor beta-1

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Abbreviations: AG, Aminoguanidine; AG/PER, aminoguanidine/perindopril; GS, glomerulosclerosis; PER, perindopril; RAS, renin-angiotensin system; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; STZ, streptozotocin; TGFβ1, transforming growth factor beta-1; TIA, tubulointerstitial area.

Based on clinical and experimental studies, the two main risk factors linked to the pathogenesis of diabetic vascular complications are hyperglycaemia and systemic hypertension [1, 2, 3, 4]. However, the interaction between these two pathways and their involvement in the pathogenesis of diabetic renal microvascular complications is not clear. Studies by our own group and others, in both the experimental and clinical setting have shown the renoprotective effects of anti-hypertensive agents that block the renin-angiotensin system (RAS) [1, 5, 6, 7]. The renal effects of these agents include reduced development of albuminuria and less glomerular and tubulointerstitial injury [8]. The effects of ACE inhibitors on renal structural injury have been mediated via the pro-sclerotic cytokine,

TGFβ1 [9]. The antialbuminuric effects of agents which interrupt the RAS could partly be mediated by actions on expression of the slit pore protein, nephrin [10]. This protein, which is expressed exclusively by glomerular epithelial cells, has been implicated in the permeability of albumin across the glomerular barrier [11].

The view that hyperglycaemia has a role in the development of diabetic nephropathy arises not only from a large body of clinical and experimental evidence highlighting the beneficial effects of intensified glycaemic control [4, 12], but also from studies exploring the glucose-dependent pathway of advanced glycation. In this biochemical process, AGEs accumulate as a result of prolonged exposure of proteins to glucose. This AGE formation involves the non-enzymatic and irreversible glycation of long-lived tissue proteins and subsequent alteration of their conformation and physiological functions [13]. We have previously reported increased concentrations of AGEs in the rat kidney after 32 weeks of streptozotocin-induced diabetes. Furthermore, a range of functional and structural parameters associated with diabetic nephropathy were ameliorated by aminoguanidine, a hydrazine-like compound that inhibits AGE formation [14]. Other studies have reported similar renoprotection with other inhibitors of AGE formation [15, 16, 17].

We investigated the potential synergistic effects of combining an agent that blocks the RAS with one that prevents the formation of AGEs. Specifically, we assessed if superior renoprotection is conferred with a combination of both agents when compared with either agent alone in the context of experimental diabetes. Following recent studies both *in vitro* [18] and *in vivo* [19] showing that ACE inhibitors can also inhibit AGE formation, we also assessed plasma and renal AGE concentrations with the various treatments. In addition, we explored the potential molecular mediators of renal functional and structural injury in diabetes by evaluating expression of the pro-sclerotic cytokine *TGFβ1*, and the slit-pore protein, nephrin.

Subjects and methods

Animal model. We used 8-week-old male spontaneously hypertensive rats (SHR), weighing 200 to 250 g housed in the Biological Research Laboratory at the Austin and Repatriation Medical Centre. The research project was approved by the Animal Welfare Committee of the Austin and Repatriation Medical Centre. Diabetes was induced by a single tail vein injection of streptozotocin (STZ) (Boehringer-Mannheim, Mannheim, Germany) at a dose of 45 mg/kg in citrate buffer after a 16-h fast [20]. Long-acting insulin (Protophane, Novo Nordisk Pharmaceuticals, Bagsvaerd, Denmark), at a dose of 4 U/day, was given to all diabetic rats by subcutaneous injection to avoid ketonuria and to promote weight gain. The rats had access to water and standard rat chow *ad libitum*.

Study groups. After inducing diabetes, the rats were randomly allocated into five groups and treated for 32 weeks as follows: diabetic SHR with no treatment (SHR-STZ), diabetic SHR treated with the AGE formation inhibitor, aminoguanidine (AG) (Fluka Chemicals, Stienheim, Switzerland) at a dose of 1 g/l in the drinking water, diabetic SHR treated with the ACE inhibitor, perindopril (PER) (Servier, Neuilly, France) at a dose of 1.25 mg·kg⁻¹·day⁻¹ in drinking water, and diabetic SHR treated with the combination of perindopril and aminoguanidine (AG/PER) at the same doses as used in monotherapy, both agents administered in the drinking water. The dose of ACE inhibition was chosen to be antihypertensive, but was not administered at the maximum dose so that potential synergistic or additive effects of AG could be determined. In addition, non-diabetic SHRs, sham injected with citrate buffer served as the control rats and were studied concurrently.

Physiological and metabolic parameters. The rats were placed in metabolic cages every 4 weeks (Iffa Credo, L'Arbessele, France) for collection of urine over 24 h to measure the albumin concentration by radioimmunoassay as described previously [14]. Systolic blood pressure (SBP) was assessed by tail cuff plethysmography in conscious, preheated rats [21] every 4 weeks. HbA_{1c} was measured by a high performance liquid chromatography method (Bio-Rad, Richmond Laboratories, Richmond, Va., USA) [22]. At week 32 the rats were killed by decapitation and their organs were harvested. The left kidney was fixed in either 4% paraformaldehyde or neutral buffered formalin for subsequent histological studies.

Glomerulosclerosis. The degree of glomerulosclerosis (GS) was evaluated by a semi-quantitative method as described previously [23]. Kidney sections were stained with haematoxylin and eosin and observed under a light microscope in a blinded fashion at a magnification of ×400 using the Imaging Analysis System (AIS, Imaging Research, St. Catherines, Ontario, Canada). Forty glomeruli from each kidney were graded according to the severity of the glomerular damage: 0, normal; 1, slight glomerular damage, the mesangial matrix and/or hyalinosis with focal adhesion, involving less than 25% of the glomerulus; 2, sclerosis of 25 to 50%; 3, sclerosis of 50 to 75%; 4, sclerosis of more than 75% of the glomerulus. The indices for glomerulosclerosis was calculated by using the following formula:

$$GS = (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4) / n_0 + n_1 + n_2 + n_3 + n_4$$

Where n_x = number of glomeruli in each grade of glomerulosclerosis.

Tubulointerstitial area. Point counting was done in the renal cortex and in the corticomedullary junction for each rat following routinely established methods [24]. In each field, 100 points were counted on a 1 cm² eyepiece graticule with 10 equidistant grid lines. A total of 12 high power fields (×400) per section were counted for each rat in all groups in the corticomedullary field. Each high power field was 0.076 mm² with 0.91 mm² being the total area counted per slide.

The percent fractional area (FA) was calculated as follows [24]:

$$\% FA = \frac{\text{number of tubulointerstitial grid intersections} \times 100}{\text{total number of grid intersections}}$$

This corresponds to the percentage of the tubulointerstitial area (TIA) in the total area counted of the section.

In situ hybridisation. The site-specific expression of *TGFβ1* mRNA was determined by in situ hybridisation, and carried out as reported [25, 26]. Briefly, four micron kidney sections were hybridised after digestion with PronaseE at 37°C. The hybridization buffer containing 2×10^4 cpm/ μ l 35 S labelled ribo-probe, 0.72 mg/ml yeast RNA, 50% deionized formamide, 100 mmol/l DTT, 10% dextran sulfate, 0.3 mol/l NaCl, 10 mmol/l Na_2HPO_4 , 10 mmol/l pH 7.5 and 5 mmol/l EDTA pH 8.0, 0.02% BSA, 0.02% Ficoll 400, and 0.02% PVP was added to each section and incubated at 60°C overnight. Following stringent washing with 50% formamide, $2 \times \text{SSC}$ at 55°C the slides were air-dried and exposed to BioMax MR film (Kodak Company, Rochester, N.Y., USA) for 3 to 5 days. Slides were coated with desiccant for a period of 2 to 4 weeks according to the autoradiography results. The slides were developed using Kodak D19 developer for 4 min, 1% acetic acid for 1 min and Ilford Hypan fixative for 4 min followed by rinsing in distilled water for at least 15 min. The sections were then fixed in 4% paraformaldehyde and treated with a progressive haematoxylin/eosin stain. Quantitation of the data was done on the renal cortex and on the corticomedullary junction from the autoradiography film. The probe binding density was determined by computerised densitometry. Before each analysis session the analysis software was densitometrically calibrated to the Linotype-Hell scanner using an AGFA transmissive greyscale step wedge. The densitometric values for the AGFA step wedge were acquired using an optically calibrated densitometer. Using a densitometrically calibrated look-up table, greyscale values were converted to optical density values.

Nephrin immunostaining. Immunohistochemistry was done according to a modified method using a specific antibody to mAb 5-1-6 antigen which is identical to rat nephrin [10, 27]. This antibody was raised in the rabbit against a synthetic peptide sequence derived from the cytoplasmic region of the nephrin protein [10, 27]. In brief, 4 μ m frozen kidney sections were cut on a cryostat at -20°C . Frozen sections were fixed with cold acetone for 10 min. Endogenous peroxidase was inactivated using 0.1% hydrogen peroxide in phosphate-buffered saline for 10 min, then sections were incubated with protein blocking agent for 20 min. Endogenous non-specific binding for biotin and avidin was blocked using a Biotin/Avidin blocking Kit (Vector Laboratories, Burlingame, Calif., USA). The kidney sections were incubated for 1 h at room temperature with mAb 5-1-6 antibody. Biotinylated goat anti-mouse immunoglobulin (Victor Laboratories, Burlingame, Calif., USA) was used as a second antibody, followed by horseradish peroxidase-conjugated streptavidin. Peroxidase activity was identified by reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical, St Louis, Mo., USA) substrate. Quantification of nephrin immunostaining was done by calculating the proportion of area occupied by the brown staining within

each glomerulus [28]. An observer who was masked to the study group of origin assessed twenty glomeruli per slide and eight slides per rat.

Measurement of AGEs

Serum levels. AGE-peptides were quantified using the flow-injection method as reported previously [19, 29]. Briefly, 20 μ l of serum was added to 480 μ l of 0.15 mol/l trichloroacetic acid (to remove protein) and 100 μ l of chloroform (to remove lipid). Twenty microlitres of the aqueous phase was then injected into the flux phase of a flow injector system and monitored in series detectors monitoring AGE-fluorescence at 440 nm (emission) and 247 nm (excitation) and measuring absorbance at 280 nm (peptide content). The results are expressed as a percent defined by a ratio of the area under the fluorescence curve divided by the area under the curve (i.e. fluorescence per peptide content) compared to that of standard AGE-peptides obtained from enzymatic hydrolysis of 10 mg/ml AGE-BSA.

Tissue levels. The concentration of total protein determined using a BCA protein assay kit (Pierce, Rockford, Ill., USA) was measured in homogenised whole kidney, previously stored at -80°C . Renal AGE peptide fluorescence was measured in 24-h acid-hydrolysed protein preparations by a flow injection system [29] with a Waters 470 spectrophotometer (370/440 nm, Waters, Milford, Mass., USA). To correct for differences in sample protein content, fluorescence intensity was expressed as arbitrary units of fluorescence per mg/collagen.

Statistics. All data are shown as means \pm SEM unless otherwise specified. Since the data for albuminuria are not normally distributed, logarithmic transformation was done prior to analysis and data are shown as geometric mean \times/\div tolerance factor (Geometric SEM). Data were initially analysed by ANOVA with post hoc comparisons between group means carried out by Fisher's least significant difference method [30] using Statview version 5.0. A *p* value of less than 0.05 was viewed as statistically significant.

Results

Metabolic parameters, blood pressure and kidney weight. Diabetes was associated with reduced body-weight gain. No effect on body weight was observed with monotherapy treatments, although the aminoguanidine and perindopril combination was associated with less body-weight gain (Table 1). HbA_{1c} was increased in all diabetic groups. Combination AG/PER

Table 1. Body weight, glycated haemoglobin and kidney weight-to-body weight ratio (KW/BW) at week 32 of the study period

Treatment	<i>n</i>	Body weight (g)	HbA_{1c} (%)	KW/BW (g/kg)
Control	15	458 \pm 9	4.7 \pm 0.4	3.3 \pm 0.1
Diabetic	16	359 \pm 9 ^a	12.3 \pm 0.3 ^a	4.5 \pm 0.1 ^a
Perindopril	17	357 \pm 5 ^a	11.9 \pm 0.2 ^a	4.4 \pm 0.2 ^a
Aminoguanidine	18	352 \pm 9 ^a	10.4 \pm 0.4 ^{a,c}	4.5 \pm 0.1 ^a
Aminoguanidine + Perindopril	16	337 \pm 6 ^{a,b}	10.3 \pm 0.4 ^{a,c}	4.7 \pm 0.1 ^a

Data are shown as means \pm SEM.

^a *p*<0.01 vs control rats; ^b *p*<0.05, ^c *p*<0.01 vs untreated diabetic rats

therapy and AG as monotherapy had a modest effect on glycated haemoglobin. Kidney enlargement was observed in the diabetic untreated rats, which was not reduced by any of the treatment groups.

Functional changes

Systolic blood pressure. Diabetes was associated with reduced SBP when compared to the control rats over the 32-week study period (ANOVA; $F=74.4$, $p<0.001$; average week 8–32 control rats 210 ± 2 vs untreated diabetic group, 185 ± 2 mmHg, $p<0.001$; Fig. 1). Perindopril reduced SBP by approximately 30 mmHg when compared to untreated diabetic rats (ANOVA; $F=117$, $p<0.001$; average week 8–32 PER 153 ± 2 mmHg $p<0.001$ vs diabetic) as did the AG/PER group, (ANOVA; $F=120$, $p<0.001$; average week 8–32 AG/PER 156 ± 2 mmHg, $p<0.001$ vs diabetic, NS vs diabetic + PER). There was no statistically significant difference in blood pressure between the PER and AG/PER treated groups. Aminoguanidine alone did not reduce SBP to any significant extent (average week 8–32, AG alone 180 ± 4 mmHg).

Albuminuria. Four weekly serial measurements of AER for week 16 to week 32 of the study period are shown in Fig. 2. Diabetes was associated with a progressive rise in albuminuria compared with non-diabetic SHR during the study period (ANOVA; $F=69.9$, $p<0.001$; week 32 diabetes $145.9 \times/\div 1.1$ mg/24 h vs control rats $48.9 \times/\div 1.2$ mg/24 h, $p<0.001$). Perindopril retarded the increase in albuminuria compared to the untreated diabetic group over the treatment period (ANOVA; $F=19.0$, $p<0.001$; week 32 PER $97.1 \times/\div 1.1$ mg/24 h, vs diabetic untreated, $p<0.001$),

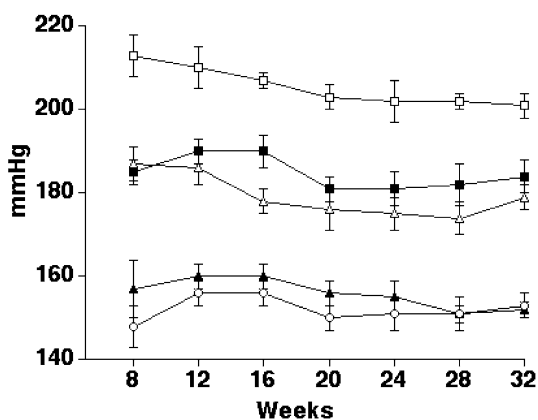


Fig. 1. Serial measurements every 4 weeks of systolic blood pressure are shown for weeks 8 to 32 of the study period for the control rats (white squares), untreated diabetic (black squares), diabetic + PER (white circles), diabetic + AG (white triangles) and diabetic + AG/PER (black triangles). Control vs diabetic rats $F=74$, $p<0.001$; diabetic + PER $F=117$, $p<0.001$; diabetic vs AG $F=2$, $p<0.1$; diabetic vs AG/PER $F=120$, $p<0.001$ ANOVA with repeated measures)

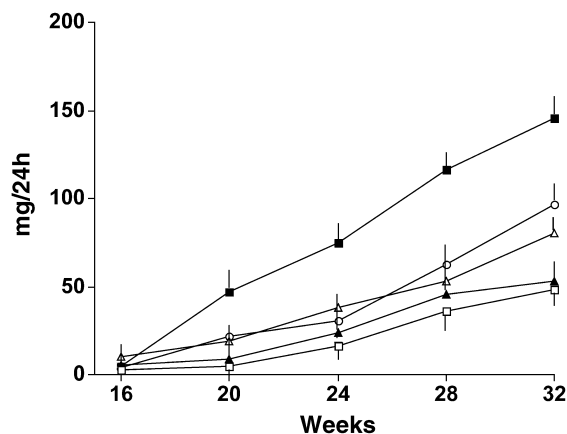


Fig. 2. Serial monthly measurements of albumin excretion rate every 4 weeks are shown for weeks 16 to 32 of the study period for the control rats (white squares), untreated diabetic rats (black squares), diabetic + PER (white circles), diabetic + AG (white triangles) and diabetic + AG/PER (black triangles). Geometric means and tolerance factors are shown. Control vs diabetic rats $F=69.9$, $p<0.001$; diabetic vs PER $F=19.0$, $p<0.001$; diabetic vs AG $F=8.6$, $p<0.01$; diabetic vs AG/PER $F=40.1$, $p<0.001$ ANOVA with repeated measures)

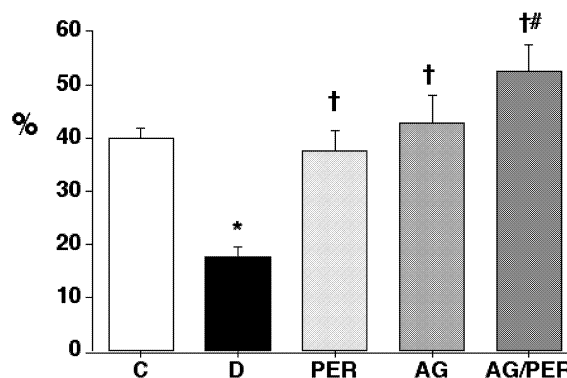


Fig. 3. Quantitation for the immunohistochemical staining for nephrin protein expression. Data are shown as means \pm SEM. C, control rats; D, diabetic untreated rats; PER, diabetic + perindopril; AG, diabetic + aminoguanidine; AG/PER, diabetic + aminoguanidine + perindopril. * $p<0.001$ vs control rats, † $p<0.001$ vs untreated diabetic rats, # $p<0.05$ vs PER

as did the aminoguanidine group (ANOVA; $F=8.6$, $p<0.01$; week 32 AG, $80.4 \times/\div 1.3$ mg/24 h, vs diabetic untreated, $p<0.01$). The combination of perindopril and aminoguanidine attenuated the development of albuminuria in the diabetic rats (ANOVA; $F=40.1$, $p<0.001$; week 32 AG/PER $59.5 \div 1.1$ mg/24 h, vs diabetic untreated $p<0.001$). This attenuation in the combination group was greater than that observed with either monotherapy treatment (AG/PER vs PER, $F=7.7$, $p<0.01$; AG/PER vs AG, $F=4.5$, $p<0.02$).

Nephryn protein expression. Diabetes was associated with a decrease in nephryn protein expression compared to the control SHR group (control, 40.0 ± 1.9 vs untreated diabetic rats, $18.6 \pm 1.9\%$; $p<0.001$) (Fig. 3).

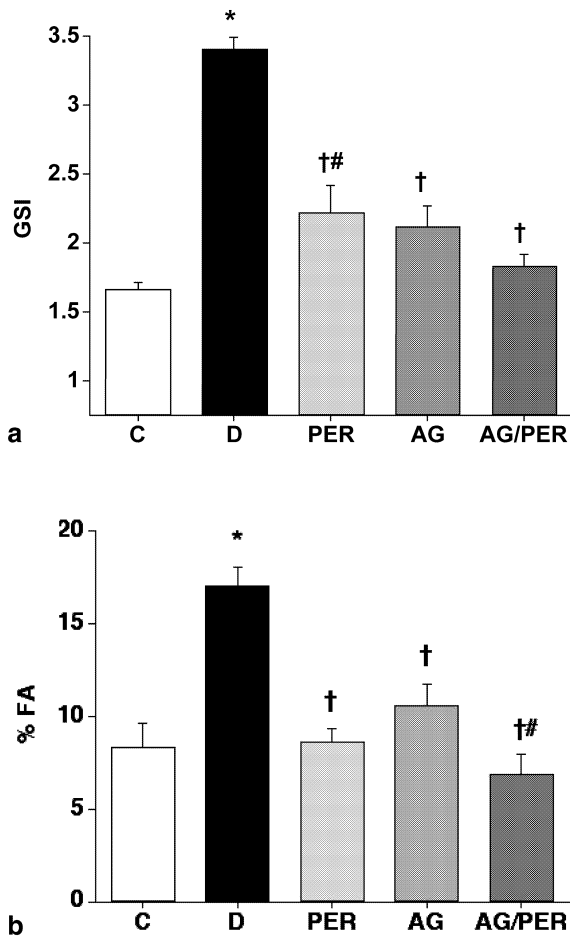


Fig. 4a, b. Glomerulosclerosis index (a) and tubulointerstitial area (b) are shown as mean \pm SEM. C—control rats; D—diabetic untreated rats; PER—diabetic + perindopril; AG—diabetic + aminoguanidine; AG/PER—diabetic + aminoguanidine + perindopril. * $p < 0.001$ vs control rats, † $p < 0.01$ vs untreated diabetic rats, # $p < 0.01$ vs AG/PER

Both PER and AG treatment groups prevented this decrease in glomerular nephrin expression (PER, $37.6 \pm 3.9\%$; AG, $42.7 \pm 5.3\%$; $p < 0.001$ vs untreated diabetic) with an additional effect observed with the combination group (AG/PER $52.6 \pm 2.5\%$, $p < 0.001$ vs untreated diabetic; $p < 0.05$ vs PER alone).

Glomerulosclerotic index (GSI). The GSI was higher in the diabetic SHR group compared to the control group (Table 1, Fig. 4a). Both AG and PER treatment groups reduced the GSI, with a further reduction in the AG/PER treated group when compared to the PER only group ($p < 0.05$).

Tubulointerstitial area (TIA). Tubulointerstitial injury was increased in the diabetic untreated group compared to the control SHR group (Table 1, Fig. 4b). There was a decrease in tubulointerstitial area in the AG and PER groups, which was further decreased by the AG/PER group compared to the AG treatment only group.

Cytokine gene expression: *TGF β 1*. Diabetes was associated with an increased expression of *TGF β 1* mRNA (Fig. 5a, b), which was normalised by all treatments including PER, AG and the combination regimen (Fig. 5b).

Quantitation of AGE concentrations: Serum AGE concentrations. Diabetes was associated with a significant increase in serum AGE concentrations compared to the control SHR (Fig. 6A). Both aminoguanidine and perindopril significantly reduced the concentration of serum AGEs compared to the untreated diabetic group. The AG/PER combination reduced the concentration of serum AGEs below that of control values although this was not significantly lower than either monotherapy.

Tissue AGE concentrations. The diabetic untreated group had an increased concentration of tissue AGE fluorescence compared to the control group (control 8.1 ± 1.4 vs untreated diabetic 14.7 ± 1.5 arbitrary units/mg protein; $p < 0.01$; Fig. 6b). This was reduced by both treatment groups as monotherapy. There was no additional reduction in AGE fluorescence afforded by the combination group treatment.

Discussion

Our study shows that the combination of an agent that interrupts the renin-angiotensin system with one that inhibits AGE formation provides superior renoprotection in a model of experimental diabetes, than either agent alone. This has been shown for a range of functional and structural parameters including albuminuria, glomerulosclerosis and tubulointerstitial injury. The results support the concept that the pathogenesis of diabetic nephropathy involves a deleterious interaction between haemodynamic and metabolic pathways [31]. These observations should be considered in the context of the beneficial effects of both ACE and AGE inhibitors on the progression of renal damage in the experimental context [5, 14, 15, 32].

It has been well established that blood pressure is a major determinant in the progression of diabetic complications, including nephropathy [3, 20]. Previous studies by many researchers including our own laboratory have shown the renoprotective effect of reducing blood pressure in retarding the progression of experimental diabetic nephropathy. As expected, treatment in this study with the ACE inhibitor, perindopril was associated with an antihypertensive and antialbuminuric effect, as has been shown with other agents that block the RAS [1, 5, 7]. It is likely that the renoprotective effect of blocking the RAS includes effects not only via specific reductions in pathways dependent on blood pressure, but also in attenuation of non-haemodynamic effects of angiotensin II. A study in this model of

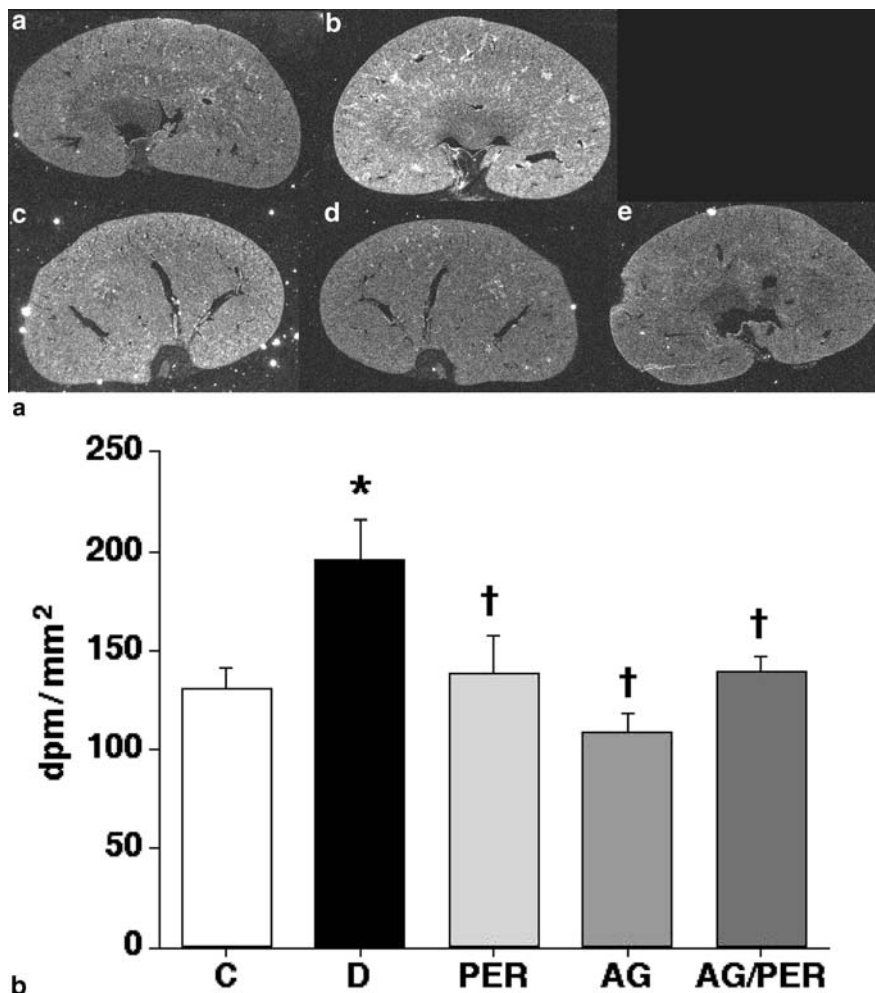


Fig. 5a,b. (a) X-ray autoradiograph film of *TGFβ1* in situ hybridisation of kidney from the control rats (a), untreated diabetic rats (b), PER (c), AG (d) and AG/PER (e). Original magnification $\times 4$. (b) Quantitation of the in situ hybridisation for the gene expression of *TGFβ1*. Data are shown as means \pm SEM. C, control rats; D, diabetic untreated rats; PER, diabetic + perindopril; AG, diabetic + aminoguanidine; AG/PER, diabetic + aminoguanidine + perindopril. * $p < 0.001$ vs control rats, † $p < 0.01$ vs untreated diabetic rats

hypertension and diabetes has shown that, despite an equihypotensive effect of the angiotensin II receptor blocker, valsartan and the dihydropyridine calcium channel blocker, amlodipine, renoprotection as assessed by reductions in albuminuria and glomerulosclerosis was afforded by valsartan only [33]. As angiotensin II (AII), the effector peptide of the RAS, has been shown to have growth-like properties [34, 35] the beneficial effects observed with perindopril, and thus the progression of albuminuria in this model, may only partly be explained by effects on systemic blood pressure. Angiotensin II has been implicated in the stimulation of transforming growth factor $\beta 1$ (TGF $\beta 1$), a prosclerotic cytokine considered pivotal in the progression of diabetic renal complications [8, 36].

Aminoguanidine treatment was also associated with an attenuation of albuminuria, despite no antihypertensive effect. The ability of the inhibition of AGE, aminoguanidine to confer antialbuminuric effects has been shown by other studies in normotensive [37] and hypertensive transgenic Ren-2 rats [38] as well as in studies involving AGE infusion [39]. However, the exact mechanism through which aminoguanidine exerts an antialbuminuric effect is not clear. It has been postulated that the dysfunction of the extracellular matrix turnover in diabetes leads to an accumulation of AGEs, resulting in mesangial expansion and basement membrane thickening, both of which are hallmarks of diabetic nephropathy [40, 41]. The ability of aminoguanidine to reduce AGE accumulation in the kidney, thus partially preserving alterations in the glomerular filtration process could account for the positive effects on albuminuria in this study. Indeed, aminoguanidine treatment was associated with a reduction in diabetes-related increases in renal AGE-fluorescence. A reduction in AGE-fluorescence was also observed in the perindopril-treated group. Previous studies by our group and others have documented the anti-AGE effect of RAS blockade both in vivo [19] and in vitro [18]. Whether this reflects the ability of AII to influence AGE production, improve renal

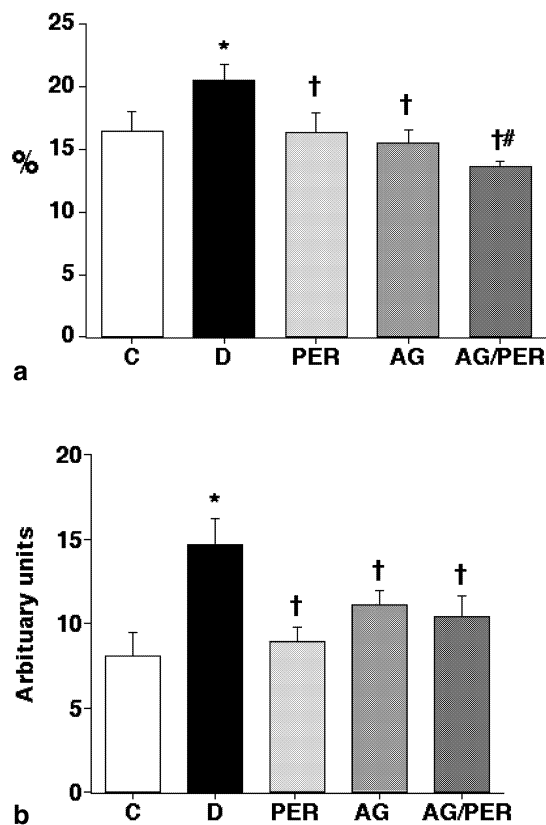


Fig. 6a, b. Quantitation of serum (a) and tissue (b) concentrations of AGEs. Data are shown as means \pm SEM. C, control rats; D, diabetic untreated rats; PER, diabetic + perindopril; AG, diabetic + aminoguanidine; AG/PER, diabetic + aminoguanidine + perindopril. * $p < 0.001$ vs control rats, † $p < 0.01$, # $p < 0.001$ vs untreated diabetic rats

clearance of AGEs or reduce tissue injury related to oxidative stress [19, 42] has not yet been determined. Increases in serum AGEs, as observed in the diabetic rats in this and other studies [15, 37, 39] have been associated with impairment of renal function [43]. However, impairment of renal function is not a prominent feature of this model [20].

It is unlikely that the renoprotective effects of AG are related to its ability to inhibit the inducible form of nitric oxide synthase. One study showed no significant changes in the progressive rise in albuminuria between AG and ALT-946, a potent inhibitor of AGE-derived protein modification that has a minimal effect on NOS inhibition [15]. In addition, other agents which inhibit AGE formation such as OPB-9195 [16] and pyridoxamine [17] retard the development of albuminuria, yet have no effect on nitric oxide synthesis. This is further suggested by our previous research showing that inhibition of NOS with L-NAME and methylguanidine failed to provide similar renoprotection to that seen with aminoguanidine [44].

In this study we observed a superior effect of combination therapy in attenuating the progression of albuminuria when compared to either agent as mono-

therapy. This was also in association with preservation of the diabetes-related depletion in nephrin, a slit-pore protein established to play a role in the glomerular filtration process [11, 45]. Our group and others have documented the prevention of nephrin depletion in both normotensive [46] and hypertensive [10, 33] models of diabetic renal injury with RAS blockade. These effects occurred in association with a concomitant reduction in the rise of albumin excretion rate. In this study we show a prevention of nephrin depletion, as assessed by immunohistochemistry, in association with both perindopril and aminoguanidine as monotherapies. There was a further increase in nephrin expression observed with the combination therapy in association with a superior effect on urinary albumin excretion. Since combination therapy was associated with the highest level of glomerular nephrin expression, and both aminoguanidine and perindopril as monotherapies preserve glomerular nephrin depletion in this model, it is likely that diabetes-associated nephrin depletion is mediated by both metabolic and haemodynamic pathways.

One potential explanation for the renoprotective effect of combination therapy could be that this treatment was associated with reduced food intake, thereby leading to reduced protein intake, a known determinant of progression of renal disease in experimental diabetes [47]. We did not use a maximal dose of ACE inhibition since higher doses of this agent, as previously reported [48], would have conferred superior renoprotection and therefore would not have allowed us to assess potential synergistic or additive effects of alternative treatments such as an inhibitor of advanced glycation.

The renal structural injury observed in the diabetic rats, as assessed in both the glomerulus and tubulointerstitium, was reduced by both treatments as monotherapy, with an additional effect observed with the combination when compared to either perindopril or aminoguanidine treatment alone. The reduction in glomerular and tubulointerstitial injury was observed in association with a reduction in the expression of the prosclerotic cytokine, TGF β 1 in all three treatment groups. Both ACE and AGE inhibition reduce the degree of glomerulosclerosis possibly via a reduction in the expression of prosclerotic cytokines such as TGF β 1 [8, 49, 50]. Our results further support to the concept that both AII and AGE formation are implicated in the progression of glomerular structural injury via a common cytokine pathway such as TGF β 1. However, as combination therapy reduced the glomerular injury more than seen with either monotherapy alone, yet no additional reduction in TGF β 1 gene expression was observed. This would imply that other factors could also be involved including growth factors such as PDGF [49] or CTGF [51] which have been shown to be AGE-dependent.

Aminoguanidine and perindopril were not only associated with a decrease in glomerulosclerosis, but

also had effects on reducing tubulointerstitial injury. The tubulointerstitium is now considered a major site of diabetes-related injury and is an important determinant of the rate of decline in renal function in diabetic nephropathy [9]. Combination therapy was associated with a further reduction in tubulointerstitial injury, and in particular was superior to aminoguanidine than to ACE inhibition. It is not clear if AII rather than AGE-dependent pathways predominate in the tubulointerstitium in diabetes. Although it has been shown in vivo and in vitro that there are AGE-mediated pathways which seem to be important in the development of tubulointerstitial injury [52], the superior effect of ACE versus AGE formation inhibition on improvement of tubular injury was recently observed in a diabetic model of hypertension and severe structural injury [38, 53]. However, in that study using the hypertensive Ren-2 model, there is local activation of the intrarenal renin-angiotensin system [53], a phenomenon not observed in the model used in our study, the SHR. Neither treatment influenced body weight, which is primarily related to tubular hypertrophy. Indeed, ACE inhibition has been reported not to affect tubular hypertrophy.

We found evidence supporting the concept that both metabolic and haemodynamic factors contribute to the development of diabetic nephropathy. It is evident that both pathways are mediating the progression of the disease and a potential common pathway could involve the pro-sclerotic cytokine, TGF β 1. The effect of the ACE inhibitor on TGF β 1 mRNA expression could be due to the reduction on AII mediated effects and also to the reduced mechanical forces as a result of reduced systemic blood pressure. However, the ability of an AGE inhibitor to reduce TGF β 1 expression without influencing systemic blood pressure, emphasises the role of non-haemodynamic effects of mediators such as AII and AGEs on TGF β expression.

This study contributes to our understanding of the multifactorial nature of diabetic nephropathy and implies that therapeutic intervention for this condition may require an approach involving different agents to inhibit the various putative pathogenic pathways. Presumably such an approach could include both AGE and ACE inhibition.

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