

Preservation of Kidney Function with Combined Inhibition of NADPH Oxidase and Angiotensin-Converting Enzyme in Diabetic Nephropathy

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Key Words

Diabetic nephropathy · Apocynin · Ramipril · NADPH oxidase

Abstract

Background/Aims: Antihypertensive therapies such as angiotensin-converting enzyme-1 inhibitors (ACEi) slow the decline in renal function seen with diabetic nephropathy, although there is still progression ultimately to end-stage renal disease. The aim of this study was to determine if there were added renoprotective benefits seen by combining ACEi with blockade of NADPH oxidase. **Methods:** Sprague-Dawley diabetic and non-diabetic rats were randomized to receive intervention therapy with apocynin (15 mg/kg/day, weeks 16–32), apocynin + the ACEi ramipril (1 mg/kg/day, weeks 16–32), or ramipril alone (1 mg/kg). **Results:** All three treatments retarded the development of albuminuria in the diabetic rats. Apocynin conferred its benefit either as a monotherapy or in combination with ramipril without affecting blood pressure per se. Renal morphological injury was attenuated by all three treatment strategies. Diabetes was associated with increasing renal fibronectin and type IV collagen protein expression, with the combination regimen resulting in the highest decrease in extracellular matrix ac-

cumulation. All three treatments prevented the diabetes-associated increases in renal cytosolic superoxide generation as well as urinary isoprostanes. While renal TGF- β 1 activation was reduced by ramipril treatment but not by apocynin as a monotherapy, kidney cortical membranous VEGF was reduced by apocynin as monotherapy and dual therapy but not by ramipril alone. **Conclusions:** Combination of NADPH oxidase blockade with ACE inhibitors is a promising regimen which warrants further investigation as a way to confer additional renoprotection in diabetes.

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Introduction

Diabetes and end-stage renal disease are increasing in incidence worldwide. Many pathophysiological mechanisms have been postulated for the onset and progression of diabetic nephropathy, including growth factors such as transforming growth factor- β (TGF- β), cytokines, activation of the renin-angiotensin system (RAS) and oxidative stress [1].

Angiotensin-converting enzyme-1 inhibitors (ACEi), such as ramipril and other blockers of RAS, provide the most effective clinical intervention to date. These inhibi-

tors are able to do this not only by lowering blood pressure but also by affording renoprotection via inhibition of the local renal RAS thereby reducing renal fibrosis [2, 3].

Oxidative stress, via increased production of reactive oxygen species (ROS), has also been implicated in the pathogenesis of diabetic nephropathy [4, 5]. The enzyme, NADPH oxidase which is a potent cytosolic generator of ROS, is composed of two membrane-associated components, p22^{phox} and gp91^{phox}, and four major cytosolic components, p47^{phox}, p40^{phox}, p67^{phox} and rac-1/2. Within the kidney, gp91^{phox} has other homologs such as nox-4 predominantly expressed in renal cortex [6]. Upregulation of specific subunits of NADPH oxidase have been demonstrated in diabetes within the kidney including p47^{phox} [7], nox-4 [8] and p22^{phox} [9]. Apocynin is a methoxy-substituted catechol which inhibits NADPH oxidase by impeding the assembly of two of the subunits, p47^{phox} and p67^{phox}, within the membrane of the NADPH oxidase complex [10, 11]. Furthermore, apocynin as monotherapy improves renal function in experimental models of diabetic nephropathy [6, 12]. The RAS has also been implicated in the production of superoxide via direct and indirect effects of angiotensin II via NADPH oxidase [13].

Therefore, this study aimed to test whether the combination of the ACEi ramipril with apocynin, an NADPH oxidase inhibitor, administered as intervention therapy once albuminuria was established, would provide greater renoprotection than monotherapy. This group of experiments addresses the structural and functional improvements provided by this combination therapy by examining various parameters such as albumin excretion rate (AER), glomerular filtration rate and accumulation of extracellular matrix proteins.

Materials and Methods

Experimental Animal Model

Experimental diabetes was induced in male Sprague-Dawley rats (200–250 g) by injection of the β -cell toxin streptozocin (50 mg/kg). Animals with a plasma glucose concentration in excess of 15 mmol/l 1 week after induction of diabetes were included in the study. Sham-injected control animals (sodium citrate buffer pH 4.5) were followed concurrently. Diabetic and control animals were randomized into groups (n = 8) which received (a) no treatment (groups C and D), and (b) the NADPH oxidase inhibitor, apocynin (15 mg/kg, 4'-hydroxy-3'-methoxy-acetophenone; Merck, Whitehouse Station, N.J., USA; groups CApo and DApo), or (c) the combination of apocynin and the ACEi ramipril (1 mg/kg; kind gift of Sanofi-Aventis; groups CApoRam and DApoRam) given orally as a late intervention from week 16 to 32. Another

diabetic group was included (group DRam) in which ramipril (1 mg/kg) was given from week 0 to 32 and this group was used as a reference group for maximal renoprotection that could be afforded by ACEi alone. Two to three units of Ultralente insulin (Ultratard HM; Novo Industries, Bagsvaerd, Denmark) were administered daily to diabetic animals to prevent ketoacidosis and improve survival. Body weight, mean systolic blood pressure by tail cuff plethysmography, glomerular filtration rate using ⁹⁹Tc-DTPA [14], AER by ELISA (Bethyl Laboratories, Montgomery, Tex., USA), and glycated hemoglobin (HbA_{1c}) [15] were measured every 8 weeks. Serum total cholesterol and triglycerides were determined by autoanalyzer (Alfred Hospital, Melbourne, Vic., Australia). All animal procedures were in accordance with guidelines established by the AMREP Ethics Committee and the National Health and Medical Research Council of Australia.

Tubulointerstitial Area

Kidney sections were stained with periodic acid Schiff stain for quantitation of tubulointerstitial area by a semiquantitative method as described previously [16]. In brief, 2- μ m kidney sections were stained with periodic acid Schiff. Tubulointerstitial area was evaluated using the point-counting technique and performed in the renal cortex of each animal according to established methods [16].

Superoxide Production

Kidneys were rapidly excised, placed in oxygen-saturated Krebs buffer and cut into \sim 1-mm³ segments [17]. The rate of NADPH-dependent superoxide anion formation in the kidney was determined by lucigenin (bis-N-methylacridinium nitrate; Sigma Chemical Company) enhanced chemiluminescence using a microtiter plate-reading luminometer (Microumat Plus, Berthold Technologies, Wildbad, Germany) [17].

Immunohistochemistry

In brief, tissue sections to be stained for fibronectin and collagen IV were digested with 0.4% pepsin for 4 min at 37°C. The primary antibodies used were rabbit anti-human fibronectin (1:4,000; DakoCytomation, Carpinteria, Calif., USA) and goat anti-human collagen IV (1:500; Southern Biotech, Birmingham, Ala., USA) [18]. Quantitation of renal cortical immunohistochemistry was performed by computer-aided densitometry (Optimus 6.5; Media Cybernetics, Silver Springs, Md., USA), as previously described [18].

TGF- β 1 ELISA

Biologically active TGF- β 1 was measured in membranous extracts of kidney cortex using the TGF- β 1 E_{max}[®] ImmunoAssay System (Promega, Madison, Wisc., USA). Membranous extracts were prepared as previously described [12]. Prior to measurement of active TGF- β 1, samples were acid-treated with 1 N HCl followed by neutralization with 1 N NaOH. Active TGF- β 1 was measured in 96-well plates by ELISA. Values are expressed as picograms per microgram of protein as determined by the BCA protein assay (Perbio Science UK, Cheshire, UK). The interassay coefficient of variation was 3.8%.

VEGF ELISA

The Quantikine Mouse ELISA kit (R&D Systems, Inc., Minneapolis, Minn., USA) was used to measure VEGF in the mem-

Table 1. Physiological and structural parameters for all rodents at week 32 (n = 8–10/group)

	Control			Diabetic			
	untreated	Apo	ApoRam	untreated	Apo	ApoRam	DRam
Plasma glucose, mmol/l	6.8 ± 0.9	6.2 ± 0.5	6.1 ± 0.6	33.2 ± 2.7*	32.3 ± 3.2*	32.5 ± 4.6*	33.8 ± 2.4*
GHb, %	5.4 ± 0.5	4.6 ± 0.6	4.0 ± 0.7	18.4 ± 2.7*	17.1 ± 1.6	14.8 ± 1.4 [†]	19.0 ± 2.7
GFR, ml/min/kg	6.6 ± 1.2	7.1 ± 1.3	6.8 ± 1.3	11.7 ± 1.0*	12.8 ± 1.0*	12.3 ± 1.1*	12.0 ± 1.7*
Total cholesterol, mmol/l	2.6 ± 0.4	2.7 ± 0.7	2.3 ± 0.4	3.2 ± 0.6 [‡]	2.6 ± 0.5 [§]	2.6 ± 0.4 [#]	3.1 ± 0.4
Triglycerides, mmol/l	2.1 ± 0.8	2.2 ± 0.5	2.1 ± 0.5	3.5 ± 1.5**	2.8 ± 0.9	2.5 ± 0.7 [§]	3.8 ± 1.6
Body weight, g	716 ± 63	734 ± 47	659 ± 50	419 ± 31*	421 ± 33*	426 ± 35*	412 ± 35*

GHb = Glycated hemoglobin; GFR = glomerular filtration rate; Apo = apocynin; Ram = ramipril. All data are mean ± SD.

* p < 0.001 vs. control group; [†] p < 0.001 vs. diabetic group; [‡] p < 0.05 vs. control group; [§] p < 0.05 vs. diabetic group; [#] p < 0.01 vs. diabetic group; ** p < 0.01 vs. control group.

branous kidney samples as per the kit instructions. Membranous samples were obtained by fractionation of kidney cortex as described previously [12]. The values of membranous VEGF are expressed as picograms per millimole of creatinine as determined by autoanalyzer (Alfred Hospital, Melbourne, Vic., Australia) [17].

8-Iso-PGF_{2α} ELISA

The Urinary Isoprostanes ELISA kit (Oxford Biomedica Research, Oxford, Mich., USA) was used to measure 15-isoprostanes F_{2t} in urine. The urine samples were diluted in an enhanced dilution buffer. The 15-isoprostanes F_{2t} in the samples or standards compete with 15-isoprostanes F_{2t} conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-isoprostanes F_{2t} coated on the microplate. The HRP activity results in color development when the substrate, TMB is added, with the intensity of the color proportional to the amount of 15-isoprostanes F_{2t}-HRP bound and inversely proportional to the amount of unconjugated 15-isoprostanes F_{2t} in the samples or standards.

Kim-1 (Rat Kidney Injury Molecule 1) ELISA

The Kim-1 ELISA kit (Cusabio Biotech Co., Ltd., Hubei, PR China) comes with a pre-coated Kim-1-specific antibody microtiter plate to which the urine samples are added. A biotinylated antibody is added followed by an avidin conjugated to HRP antibody. TMB is then added to the plate for a color reaction which is measured spectrophotometrically at a wavelength of 450 nm.

Statistical Analysis

Results are expressed as mean ± standard deviation unless otherwise specified. Data for albuminuria were not normally distributed and therefore analyzed following logarithmic transformation. Analyses were performed by ANOVA followed by post hoc analysis using Tukey's least significant difference method, correcting for multiple comparisons. Student's t test was performed for analyses of some diabetic versus diabetic plus treatment groups as required. A value for p < 0.05 was considered to be statistically significant.

Results

Physiological and Structural Parameters

Diabetes was associated with decreased body weight in all groups (table 1), which was not altered by any treatment regimen. Diabetic rodents had elevations in plasma glucose concentrations which were unaffected by any treatment (table 1). Glycated hemoglobin was significantly increased by diabetes (table 1), and modestly reduced by combination therapy yet remained markedly elevated with this treatment regimen.

Plasma total cholesterol was modestly elevated in diabetic rodents and significantly reduced by both apocynin either as monotherapy or in combination with ramipril (table 1). Triglycerides were also increased in diabetic rodents (table 1); however, only the combination treatment led to a significant, albeit modest decrease in triglycerides. Ramipril monotherapy did not influence any lipid parameters.

Twenty-four-hour AER (fig. 1a) was elevated in diabetic rats and this increase was prevented to a greater degree by combination therapy or ramipril therapy when compared to apocynin monotherapy. Glomerular filtration rate (table 1) was increased in diabetic rats but was not affected by any other treatments. There was a modest increase in blood pressure in the diabetic rats, which was significantly reduced by both monotherapy and by combination therapy (fig. 1b).

Oxidative Stress

NADPH oxidase-dependent superoxide generation was greatly increased in renal tissues from diabetic rats with all treatments resulting in a significant decrease in

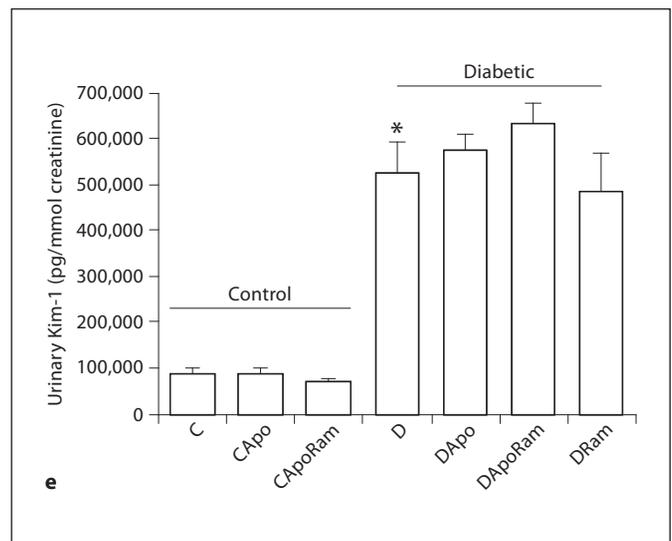
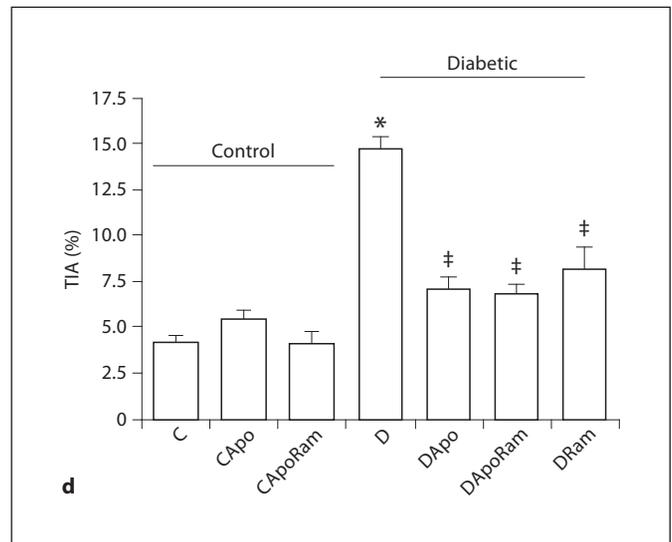
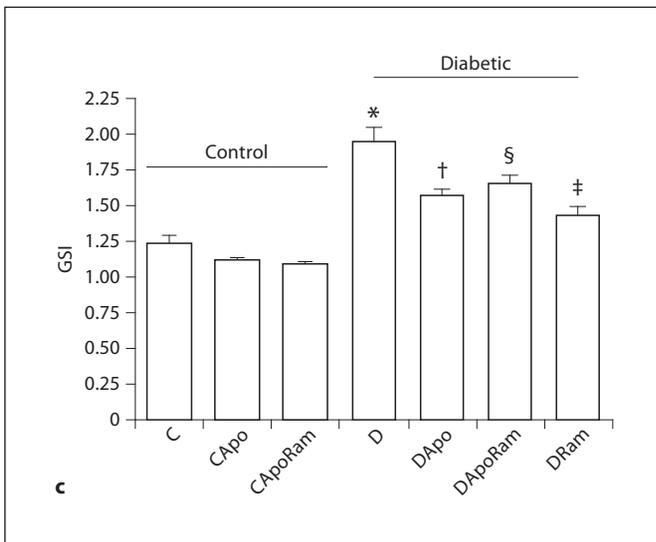
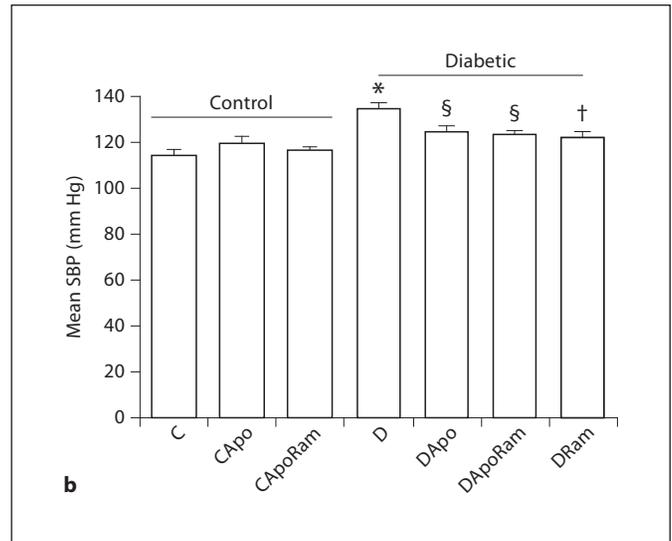
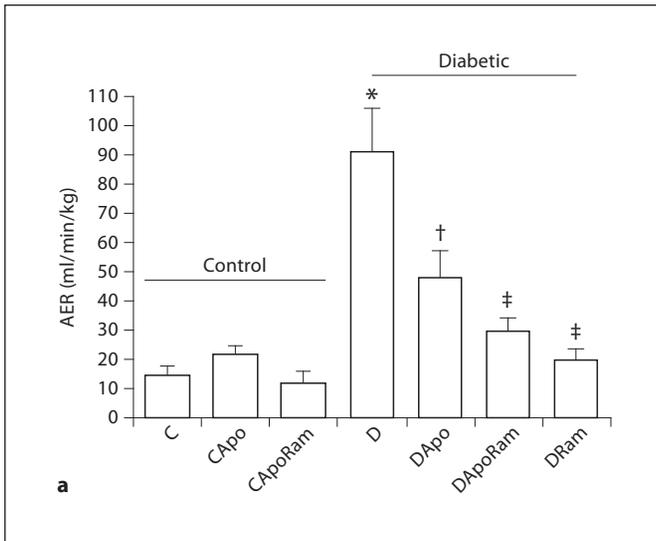


Fig. 1. Renal functional data at week 32 (n = 8–10/group). **a** Albumin excretion rate (AER) as determined by ELISA in 24-hour urine collections, geometric mean \times/\div tolerance factors shown. **b** Mean SBP as determined by tail cuff plethysmography. **c** Glomerulosclerotic index (GSI). **d** Tubulointerstitial area (TIA; %). **e** Rat Kidney Injury Molecule-1 (Kim-1) as determined by ELISA. * p < 0.001 vs. control group; † p < 0.01 vs. diabetic group; ‡ p < 0.001 vs. diabetic group; § p < 0.05 vs. diabetic group.

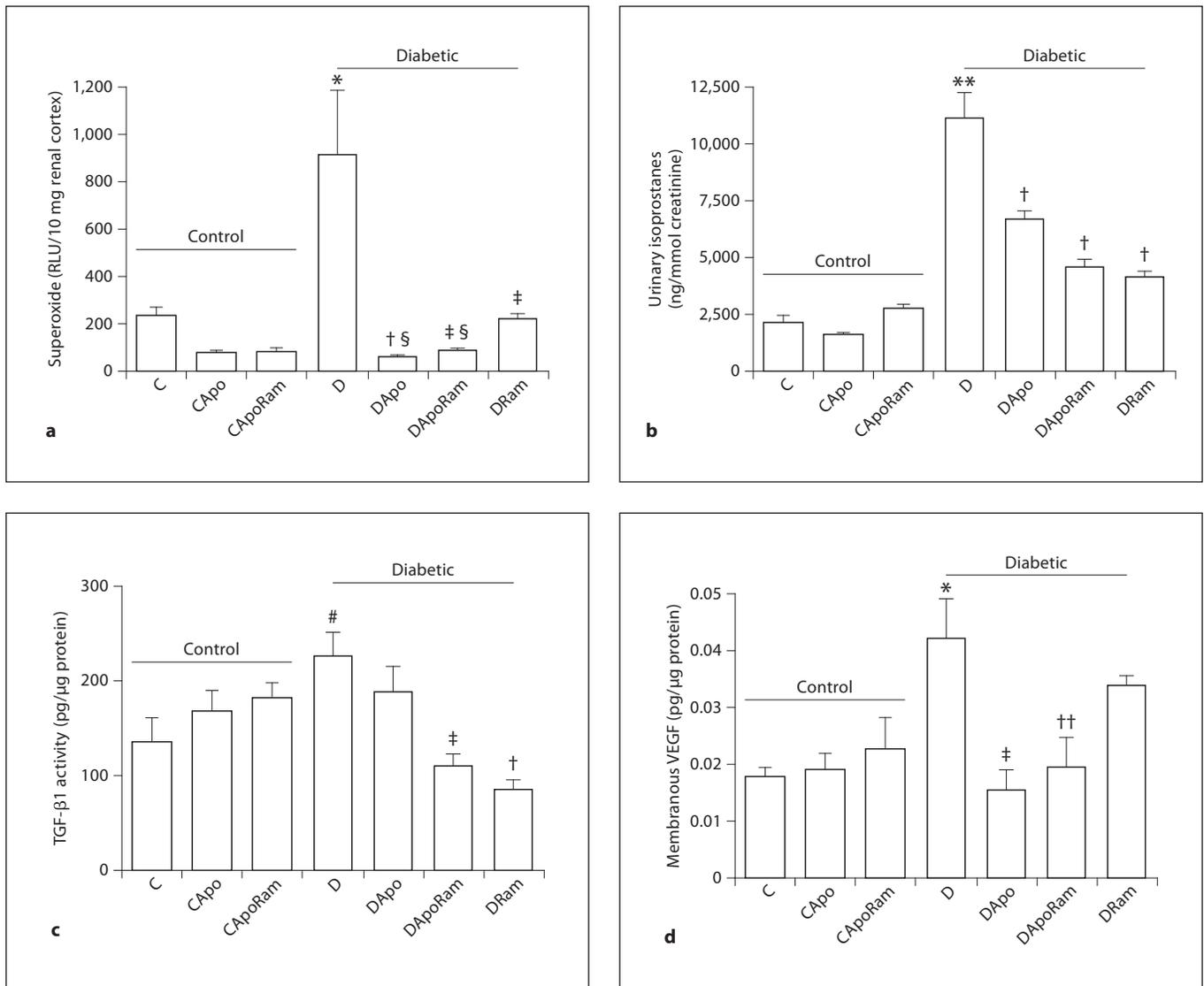


Fig. 2. Various renal parameters at week 32 (n = 8–10/group). **a** NADPH oxidase-dependent superoxide production by enhanced chemiluminescence. **b** Urinary isoprostanes by ELISA. **c** Biologically active TGF- β 1 activity. **d** Renal cortical membranous VEGF. * p < 0.01 vs. control group; † p < 0.001 vs. diabetic group; ‡ p < 0.01 vs. diabetic group; § p < 0.01 vs. diabetic plus ramipril group; ** p < 0.001 vs. control; # p < 0.05 vs. control group; †† p < 0.05 vs. diabetic group.

this parameter of oxidative stress (fig. 2a). Furthermore, apocynin was more effective than ramipril at reducing renal cytosolic superoxide production with the combination more effective than ramipril alone but not apocynin as a monotherapy. Urinary isoprostanes (15-isoprostanes F_{2t}) were also increased by diabetes and all three treatments were able to attenuate this increase.

Diabetes-Induced Increases in Extracellular Matrix Proteins

Mesangial expansion was increased with diabetes as measured by the glomerulosclerotic index (GSI) and attenuated with all treatments (fig. 1c). Tubulointerstitial area, a marker of renal structural injury was also significantly increased by diabetes and decreased by all treatments (fig. 1d). Measurement of Kim-1 in the rat urine resulted in increased expression in diabetic animals;

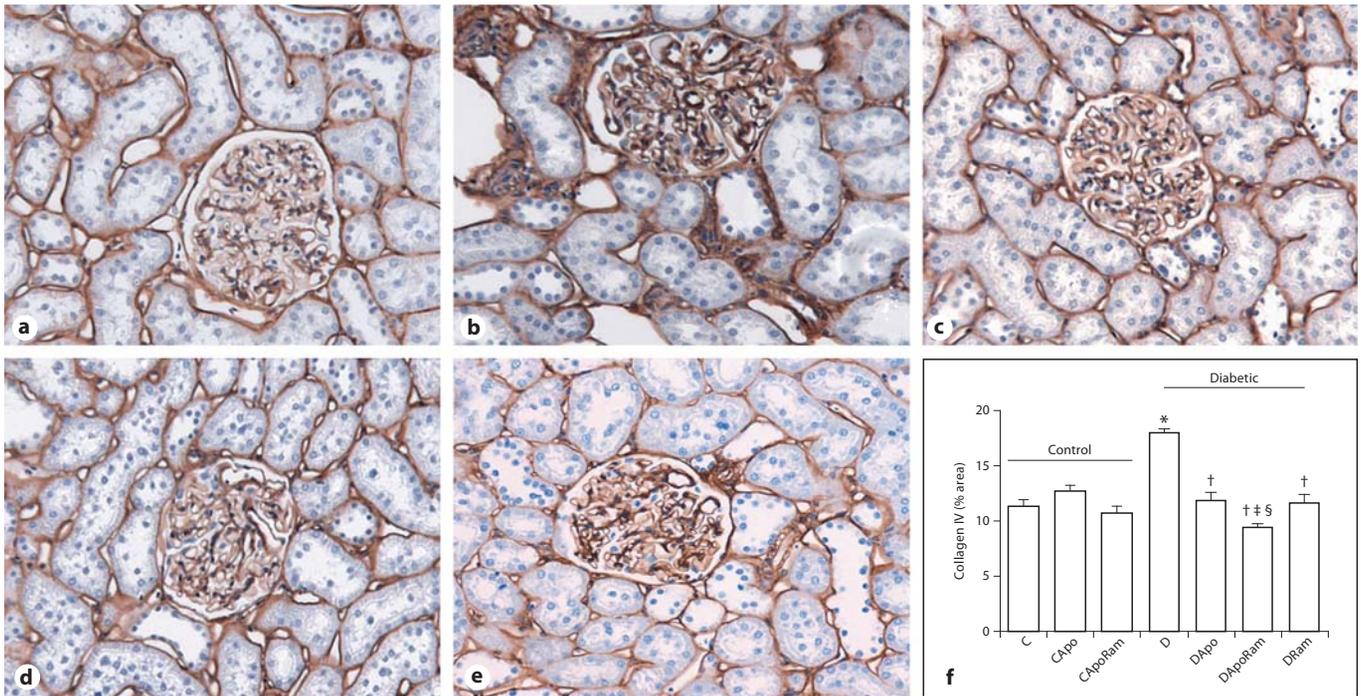


Fig. 3. **a** Computer-aided analysis of renal cortical collagen IV at 32 weeks expressed as percent area. **a–e** Representative photomicrographs of collagen IV immunostaining in kidney glomeruli of 32-week-old rats ($\times 400$ magnification). **a** Control kidney. **b** Diabetic kidney. **c** Diabetic plus apocynin kidney. **d** Diabetic plus apocynin and ramipril kidney. **e** Diabetic plus ramipril kidney. **f** Computer-aided image analysis of renal glomerular collagen IV immunohistochemistry (% area) at 32 weeks. * $p < 0.001$ vs. control group; † $p < 0.001$ vs. diabetic group; ‡ $p < 0.005$ vs. diabetic plus apocynin group; § $p < 0.01$ vs. diabetic plus ramipril group.

however, this increase was not reduced by any of the treatment strategies.

Diabetic rat kidneys also had an approximately 100% increase in biologically active TGF- $\beta 1$, an important cytokine in diabetic nephropathy, which was decreased to levels equal to or lower than that seen in control rats with combination therapy or ramipril treatment but not with apocynin monotherapy (fig. 2b). Membranous VEGF was increased 2-fold by diabetes and this increase was attenuated by apocynin monotherapy or in combination with ramipril. Ramipril monotherapy was able to reduce VEGF, but this reduction did not reach statistical significance. By immunohistochemistry, collagen IV was localized to the glomerular basement membrane, as well as the Bowman's capsules and tubular basement membranes. The expression of renal cortical glomerular collagen IV was increased almost 2-fold with diabetes. This increase in expression was attenuated with apocynin or ramipril alone. However, there was a significantly greater attenuation of collagen IV protein expression in the apocynin/

ramipril combination therapy group (fig. 3). Fibronectin staining was localized to the mesangium in the peripheral capillary wall and Bowman's capsule. Renal glomerular fibronectin was significantly increased in diabetic kidneys and reduced with either treatment regimen. Furthermore, the combination treatment and ramipril alone appeared to be more effective than apocynin alone in reducing glomerular fibronectin expression (fig. 4).

Discussion

In the present study, we have examined the therapeutic potential of apocynin, an NADPH oxidase assembly inhibitor, in experimental diabetic nephropathy alone and in combination with the most widely used clinical intervention for diabetic nephropathy, an ACEi, ramipril [19]. Our primary aim was to determine whether combination therapy would provide superior renal functional and structural protection in an experimental model of dia-

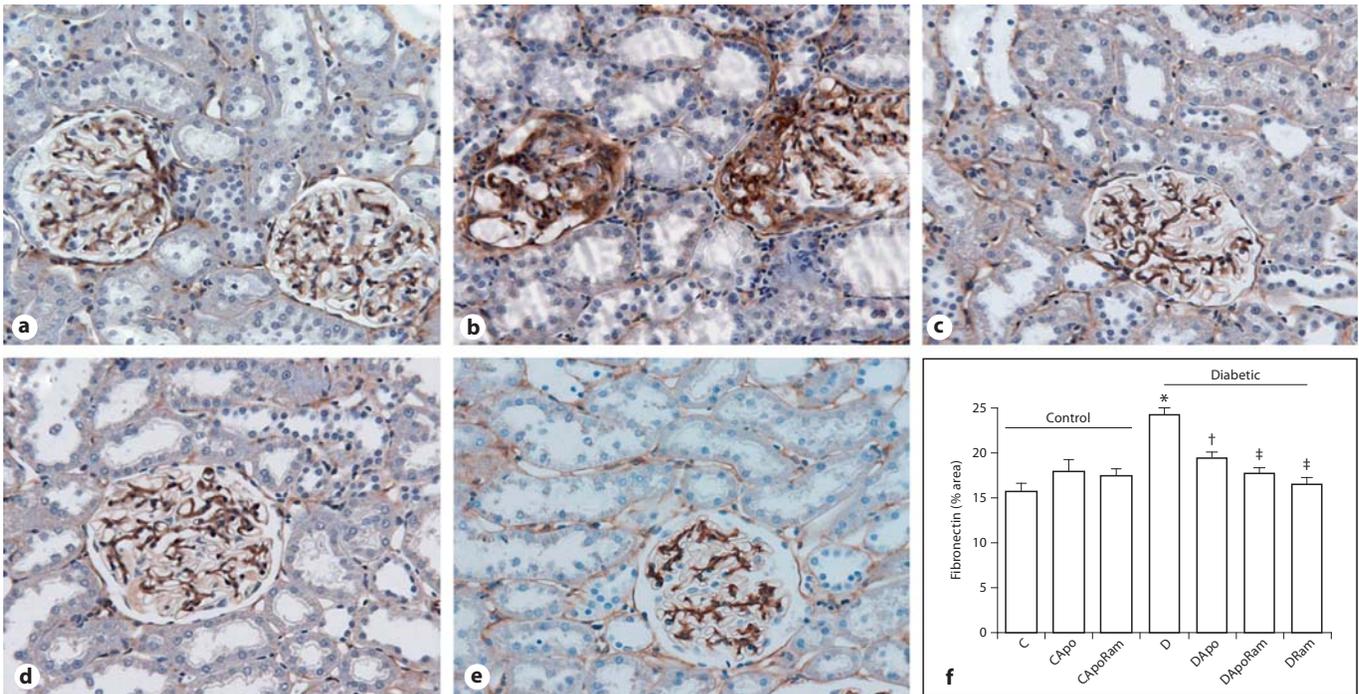


Fig. 4. Morphometric quantification of the extracellular matrix protein fibronectin. **a–e** Representative photomicrographs of fibronectin immunostaining, in kidney glomeruli of 32-week-old rats ($\times 400$ magnification). **a** Control kidney. **b** Diabetic kidney. **c** Diabetic plus apocynin kidney. **d** Diabetic plus apocynin and ramipril kidney. **e** Diabetic plus ramipril kidney. **f** Computer-aided image analysis of renal glomerular fibronectin immunohistochemistry (% area) at 32 weeks. * $p < 0.001$ vs. control group; † $p < 0.01$ vs. diabetic group; ‡ $p < 0.001$ vs. diabetic group.

betic nephropathy. Our studies identified a number of factors that were significantly improved by the combination therapy, although most renal parameters were affected to a similar extent to that seen with ramipril treatment alone. Specifically, renal functional parameters such as AER as well as mean systolic blood pressure were improved by combination therapy, although these changes were equivalent to that seen with ramipril treatment alone. Interestingly, diabetes-induced increases in renal cortical collagen IV were more significantly reduced by combination therapy than by apocynin or ramipril monotherapy. However, this did not translate to superiority in terms of renal morphological injury including parameters such as tubulointerstitial area and GSI.

Many studies have demonstrated significant improvements in urinary AERs in animals treated with ACEi [20]. Previously, Asaba et al. [21] reported that apocynin decreased proteinuria in a relatively shorter study of only 8 weeks of diabetes. Our own group has also demonstrated that chronic apocynin treatment for 16 weeks from the

time of induction of diabetes also prevented the development of albuminuria in diabetic rats [12]. In the present study, apocynin administered as an intervention therapy from 16 to 32 weeks was able to significantly decrease albuminuria after the development of early renal changes such as microalbuminuria were already evident. In combination with ramipril, there was a further decrease in AER similar to that seen with ramipril alone. It remains to be determined if longer duration studies would clearly slow the superiority of this combination on renal functional parameters such as albuminuria. Even though apocynin did not appear to have an additive effect on AER, urinary isoprostanes were decreased by all treatments in a similar pattern. This suggests that apocynin is able to decrease oxidative stress in the urine; however, there is no further benefit above ramipril treatment alone.

Tubular Kim-1 expression is specific to ongoing tubular cell damage and dedifferentiation and thus urinary levels may reflect this expression [22]. Urinary Kim-1, a marker of tubular injury, did not correlate with AER; this

was also shown by van Timmeren et al. [22] in a human study suggesting that AER is not a good marker of ongoing kidney injury in diabetes. Furthermore, it has been shown that blocking the RAS at an earlier time point in diabetic nephropathy does not improve ongoing renal damage [23]. Urinary Kim-1 has also been shown to be correlated with renal inflammation [22], and thus, as the disease is continuing, there is constant triggering of inflammatory cells and this could account for the increased expression with all three treatment regimes.

The overproduction of mesangial matrix seen in diabetic renal disease is likely to occur partly as a result of the effect of intraglomerular hemodynamic changes such as glomerular hypertension promoting the synthesis of prosclerotic cytokines such as TGF- β 1 [24, 25]. This glomerular hypertension appears to occur in part as a result of the intrarenal actions of angiotensin II, the major effector peptide of the RAS. ACEi have been demonstrated to reverse intraglomerular hypertension seen in diabetes with this considered one of the putative mechanisms for the renoprotection conferred by this class of antihypertensive agent. However, this must be considered in the context that these agents also influence systemic blood pressure. Apocynin has also been shown to significantly decrease blood pressure in a study on DOCA-salt rats [26]. In this study apocynin, as reported previously, has an impact on blood pressure in these rats and one cannot exclude an effect of this agent on intrarenal hemodynamics.

Interestingly, there was a disparate effect on renal TGF- β 1 activation between ramipril and apocynin treatment. Whereas ACE inhibition reduced TGF- β 1 activation, consistent with previous reports examining TGF- β gene and protein [27] expression, apocynin did not as a monotherapy affect this parameter. This would suggest that angiotensin II is a particularly relevant stimulus for TGF- β 1 activation in diabetes, consistent with a large body of in vitro data in various cell populations linking angiotensin II directly to TGF- β 1 expression and activation [3, 28]. Although ROS have been linked to TGF- β 1 activation [29], they may be less potent as stimuli for oxidative stress in the in vivo context of diabetes.

A number of pathways including ROS [30] and TGF- β 1 [25] have been implicated in the downstream expression of extracellular matrix proteins such as fibronectin in diabetic complications [31, 32]. Within the current study, inhibition of NADPH oxidase with subsequent reductions in cytosolic superoxide, ameliorated the diabetes-dependent increases in fibronectin, although there was no added benefit seen using combination with

ramipril suggesting that both apocynin and ramipril may decrease fibronectin accumulation via a common pathway. Expression of the extracellular matrix protein fibronectin has previously been shown to be decreased by treatment with apocynin [12, 21]. Indeed, in the present study, inhibition of NADPH oxidase by apocynin was associated with decreased fibronectin expression, and this was further improved by combination therapy with ramipril, although this was not superior to ramipril therapy.

Collagen IV is another important extracellular matrix protein which may be influenced by the pathways activated in the present study. Many groups have shown that mesangial cells grown in high glucose have increased collagen IV and that this increased expression is linked to ROS generation [33, 34]. Previous studies have shown beneficial effects of treatment of experimental diabetes with ramipril [27] or apocynin [12] monotherapies in decreasing collagen IV expression; however, the combination of these two therapies has not been described previously. In the present study, although expression of collagen IV in the diabetic kidneys was reduced by both monotherapies, combination therapy led to a more significant decline in renal collagen IV expression. However, kidney cortical membranous VEGF expression was decreased by apocynin monotherapy or combination therapy, whereas ramipril alone had no effect. Interestingly, tubulointerstitial area and GSI were reduced to a similar extent by all treatments. As previously shown, TGF- β signaling is involved in the accumulation of extracellular mesangial matrix proteins in the kidney; however, VEGF has been implicated as a mediator of diabetic albuminuria and proteinuria thus suggesting that there are two different pathways working in the thickening of glomerular basement membrane with altered matrix composition [35]. In OLETF rats, apocynin has been shown to be effective in preventing the progression of diabetic nephropathy as it reduces the glomerular and mesangial expansion by inhibiting renal VEGF expression [36]. This could explain the discrepancies between the various treatments in relation to collagen IV deposition.

In our study we found that certain lipid parameters, such as cholesterol and triglycerides, were only improved by the combination regimen. The underlying cause for this effect is unknown with both agents not considered to be lipid-lowering agents per se. It is possible that the improved lipids are a manifestation of a renal benefit of this treatment strategy with previous clinical and preclinical studies linking reduction in proteinuria and improved renal function to improved lipid profiles [37].

The present study has demonstrated that two different strategies, interruption of the RAS and ROS inhibition presumably via NADPH oxidase inhibition, confer renoprotection in a well-defined model of diabetic nephropathy. However, the failure of this combination to confer additional benefits on a range of renal parameters would suggest that angiotensin II and ROS are activating similar pathways. Nevertheless, the combination had some additional benefits, particularly on renal collagen IV accumulation. Thus, this combination strategy, possibly explored in a more advanced model for a longer period, could be a promising therapeutic approach to reduce the burden and manifestations of diabetic nephropathy.

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