

Attenuation of tubular apoptosis by blockade of the renin-angiotensin system in diabetic *Ren-2* rats

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Background. Tubular atrophy is a major feature of most renal diseases and is closely associated with loss of renal function. The present study sought to investigate tubular epithelial cell apoptosis in experimental diabetic nephropathy and to explore the role of pro-apoptotic [transforming growth factor- β (TGF- β) and anti-apoptotic growth factors [epidermal growth factor (EGF)]. The effects of renoprotective therapy with blockade of the renin-angiotensin system (RAS) also were examined.

Methods. Six-week-old female *Ren-2* rats were injected with streptozotocin (STZ) and maintained diabetic for 12 weeks. Further groups of diabetic rats were treated with the angiotensin-converting enzyme (ACE) inhibitor, perindopril, or the angiotensin II type 1 (AT1) receptor antagonist, valsartan, for 12 weeks.

Results. Widespread apoptosis, identified immunohistochemically by single stranded DNA and TUNEL, was noted in the tubules of diabetic *Ren-2* rats. These changes were associated with a 50% decrease in EGF expression and a twofold increase in TGF- β 1 mRNA. Treatment of diabetic *Ren-2* rats with either valsartan (20 mg/kg/day) or perindopril (6 mg/kg/day) reduced apoptosis to control levels in association with supranormal levels of EGF mRNA ($P < 0.01$) and a reduction in TGF- β 1 gene expression ($P < 0.05$) to that of control rats.

Conclusions. Tubular apoptosis is a prominent feature of diabetic *Ren-2* rats that is attenuated by blockade of the RAS in association with modulation of pro- and anti-apoptotic growth factor expression.

Tubulointerstitial injury, characterized by interstitial fibrosis and tubular atrophy, is a major feature of most renal diseases including diabetic nephropathy [1]. Both interstitial fibrosis and tubular atrophy are closely associated with loss of renal function [2]. While the mechanisms underlying the development of interstitial fibrosis

have been the subject of investigation, the pathogenesis of tubular atrophy remains poorly understood. Recently, several studies have suggested that apoptosis rather than necrosis may account for tubular atrophy in human as well as experimental chronic renal disease [3, 4].

Certain renal growth factors have been pathogenetically linked to disease progression and apoptosis while others may protect cells from injury. For instance, transforming growth factor- β (TGF- β) has been consistently implicated in the induction of both glomerulosclerosis and tubulointerstitial fibrosis in a wide range of renal diseases including diabetic nephropathy [5, 6]. In addition, to its pro-fibrotic effects TGF- β also may contribute to renal disease progression by its potent pro-apoptotic activity [7]. In contrast, epidermal growth factor (EGF) is a renoprotective growth factor that is implicated more in the recovery from renal injury and the protection from apoptotic cell death [8, 9].

Blockade of the renin-angiotensin system (RAS) is a major therapeutic strategy in both diabetic [10] and non-diabetic renal disease [11], with evidence of effects beyond amelioration of systemic and intraglomerular hypertension [12]. Indeed, in addition to their beneficial effects on renal function, angiotensin-converting enzyme (ACE) inhibitors and angiotensin-II type 1 (AT1) receptor antagonists also have been shown to reduce tubular injury in inflammatory, metabolic [13], hemodynamic [14] and mechanically induced renal injury [15].

The transgenic (mRen-2)²⁷ rat with the mouse *Ren-2* gene inserted into the genome of a Sprague-Dawley rat overexpresses renin and angiotensin II (Ang II) at sites of normal physiological expression [16]. When rendered diabetic, the *Ren-2* rat develops advanced glomerulosclerosis and tubulointerstitial disease with concomitant renal impairment [5, 17]. Indeed, as in diabetic kidney disease in humans, blockade of the RAS in this model provides renoprotective effects beyond those due to blood pressure reduction alone [5, 17], which makes it a useful

Key words: cell death, transgenic rats, diabetic nephropathy, tubulointerstitial disease, growth factors, renoprotection.

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tool to examine the cellular mechanisms responsible for the progression of diabetic nephropathy.

In the present study, we sought to investigate tubular epithelial cell apoptosis in relation to expression of both pro- and anti-apoptotic growth factors in experimental diabetic nephropathy and to examine the effects of RAS blockade on these parameters.

METHODS

Animals

Six-week-old female, heterozygous *Ren-2* rats, weighing 125 ± 5 g were randomized to receive either 55 mg/kg of streptozotocin (STZ; Sigma, St. Louis, MO, USA) diluted in 0.1 mol/L citrate buffer, pH 4.5 (diabetic) or citrate buffer alone (non-diabetic) by tail vein injection following an overnight fast [17]. Female rats were studied because they do not require maintenance antihypertensive therapy and hence have a lower systolic blood pressure. Diabetic and non-diabetic *Ren-2* rats ($N = 6$ per group) were treated either with an ACE inhibitor, perindopril (Servier Laboratories, Paris, France; average dose, 6 mg/kg/day), or an AT1 receptor antagonist, valsartan (20 mg/kg/day), for 12 weeks post-STZ, or vehicle. All rats were housed in a stable environment (maintained at $22 \pm 1^\circ\text{C}$ with a 12-hour light/day cycle) and allowed free access to tap water and standard rat chow containing 0.25% Na^+ and 0.76% K^+ (GR2; Clark-King & Co., Gladesville, NSW, Australia). Each week, rats were weighed and blood glucose (non-diabetic, 4 to 8 mmol/L; diabetic, 18 to 25 mmol/L) was determined using an AMES glucometer (Bayer Diagnostics, Melbourne, Australia). Diabetic rats received a daily injection of insulin (2 to 4 units IP; Ultratard, Novo Nordisk, Denmark) to maintain an average daily blood glucose of between 18 and 25 mmol/L, to promote weight gain, reduce ketoacidosis and reduce mortality [17]. Experimental procedures adhered to the guidelines of the National Health and Medical Research Council of Australia's Code for the Care and Use of Animals for Scientific Purposes and were approved by the Bioethics Committee of the University of Melbourne.

Tissue preparation

Rats were anesthetized (Nembutal, 60 mg/kg body wt IP; Boehringer-Ingelheim, Parramatta, NSW, Australia) and the abdominal aorta cannulated with an 18 G needle. Perfusion-exsanguination commenced at 180 to 220 mm Hg via the abdominal aorta with 0.1 mol/L phosphate buffered saline (PBS), pH 7.4 (20 to 50 mL) to remove circulating blood and the inferior vena cava adjacent to the renal vein was simultaneously severed allowing free flow of the perfusate. After clearance of circulating blood, 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4 was perfused for a further five minutes (100 to

200 mL of fixative) to fix the tissues [17]. Kidneys were removed from the animal, decapsulated and sliced transversely. Kidneys were post-fixed in the same fixative for two hours, routinely processed, embedded in paraffin and sectioned.

Histopathology

Tubular pathology was assessed on kidney sections stained with a modified Masson's trichrome stain [17]. Tubular atrophy and fibrosis were quantified in trichrome stained kidney section as previously described [5, 14]. In brief, under high power magnification ($\times 200$) five random and non-overlapping fields from each slide ($N = 6$ /group) were captured and digitized using a Fujix HC-2000 digital camera (Fuji, Tokyo, Japan). The relative percentage of atrophic tubules and fibrosis (blue stain) was quantitatively measured [18], using computerized image analysis (AIS, Ontario, Canada).

Immunohistochemistry

Three-micron sections were placed into histosol to remove the paraffin wax, hydrated in graded ethanol, and immersed into tap water before being incubated for 20 minutes with normal goat serum (NGS) diluted 1:10 with 0.1 mol/L PBS at pH 7.4. Sections were then incubated for 18 hours at 4°C with specific monoclonal antisera to single-stranded DNA (ssDNA; Alexis Biochemicals, San Diego, CA, USA) to evaluate apoptosis. Sections incubated with 1:10 NGS instead of the primary antiserum served as the negative control. After thorough washing with PBS (3×5 min changes), the sections were flooded with a solution of 5% hydrogen peroxide, rinsed with PBS (2×5 min), and incubated with biotinylated goat anti-rabbit IgG (Dakopatts, Glostrup, Denmark) diluted 1:200 with PBS. Sections were rinsed with PBS (2×5 min) and incubated with an avidin-biotin peroxidase complex (Vector, Burlingame, CA, USA) diluted 1:200 with PBS. Following rinsing with PBS (2×5 min), sections were incubated with 0.05% diaminobenzidine and 0.05% hydrogen peroxide (Pierce, Rockford, IL, USA) in PBS at pH 7.6 for one to three minutes, rinsed in tap water for five minutes, counterstained in Mayer's hematoxylin, differentiated in Scott's tap water, dehydrated, cleared and mounted in Depex.

TUNEL

Cell death was identified by 3' in situ end labeling of fragmented DNA with biotinylated deoxyuridine-triphosphate (dUTP) as previously described [19].

In brief, sections of formalin fixed tissue were dewaxed and hydrated. Following digestion with Proteinase K (2 $\mu\text{g}/\text{mL}$; Sigma), sections were consecutively washed in TdT buffer (0.5 mol/L cacodylate pH 6.8, 1 mmol/L cobalt chloride, 0.15 mol/L NaCl) and then incubated at 37°C with TdT (25 U, Boehringer-Mannheim, Mann-

heim, Germany) and biotinylated dUTP (1 nmol/ μ L). After washing in TB buffer (300 mmol/L NaCl, 30 mmol/L sodium citrate) to terminate the reaction, incorporation of biotinylated dUTP was detected by a modification of the ABC method as previously reported. A mouse spleen with increased apoptosis was used as a positive control for TUNEL. The omission of TdT during dUTP nick end labeling provided a negative control [19].

In situ hybridization

A 985 bp cDNA coding for rat TGF- β 1 (gift of Dr. Qian, Bethesda, MD, USA) was cloned into pBluescript KS+ (Stratagene Inc., La Jolla, CA, USA). The cDNA was then linearized with XbaI and an antisense probe was generated using T3 RNA polymerase. The 400 bp cDNA probe for EGF was cloned into pGEM 3Z (Promega, Madison, WI, USA) and linearized with HindIII to produce an antisense riboprobe (NEN, Boston, MA, USA) using T7 RNA polymerase. Antisense riboprobes were then labeled with [32 P]UTP and in situ hybridization was performed as previously described. In brief, tissue sections were dewaxed, rehydrated, and then digested with Pronase E [125 μ g/mL in 50 mmol/L Tris-HCl, pH 7.2, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0] at 37°C for 10 minutes. After 2 \times 2 minutes \times rinses in 0.1 mol/L phosphate buffer, pH 7.2, sections were fixed in 4% paraformaldehyde, pH 7.4 for 10 minutes at room temperature and rinsed again in 0.1 mol/L phosphate buffer. Sections were then dehydrated through graded ethanol and air-dried. Sections were hybridized with 32 P-labeled anti-sense EGF and TGF- β 1 probes (5 \times 10⁵ cpm/25 μ L hybridization buffer) in hybridization buffer (300 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5, 10 mmol/L Na₂HPO₄, pH 6.8, 5 mmol/L EDTA, pH 8.0, 1 \times Denhardt's solution, 0.8 mg yeast RNA/mL, 50% deionized formamide and 10% dextran sulfate), heated to 85°C and 25 μ L added to the sections. Sections hybridized with sense probe for EGF and TGF- β 1 were used as controls for non-specific binding. Coverslips were placed on the sections and the slides incubated in a humidified chamber (50% formamide/2 \times SSC) at 60°C for 14 to 16 hours. Slides were then washed in 2 \times SSC (0.3 mol/L NaCl, 0.33 mol/L Na₃C₆H₅O₇·2H₂O) containing 50% formamide at 50°C to remove the coverslips. The slides were again washed with 2 \times SSC, 50% formamide for a further one hour at 55°C. Sections were then rinsed three times in RNase buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, pH 8.0, 0.5 mol/L NaCl) at 37°C and treated with 150 μ g RNase A/mL in RNase buffer for a further one hour at 37°C, then washed with 2 \times SSC at 55°C for 45 minutes. Finally, sections were dehydrated through graded ethanol, air dried and exposed to Kodak Biomax MR Autoradiography film (Eastman Kodak, Rochester, NY, USA) for four days at room temperature. Slides were coated with Ilford K5 emulsion (1:1 with 2% gly-

erol in distilled water; Ilford, Moberly, Cheshire, UK), stored with desiccant at room temperature for 21 days, developed in Ilford phenisol, fixed in Ilford Hypam and stained with hematoxylin and eosin (H&E).

Quantitation of immunohistochemistry and in situ hybridization

The levels of gene expression of EGF and TGF- β were assessed by two different methods. The first approach considered the kidney as separate cortical and medullary components and involved densitometric evaluation of autoradiographic images. The second approach explored the possibility that there were cell specific changes in response to diabetes by quantitating the area of autoradiographic grains within specific components of the kidney [18]. Tissue sections from all animals were exposed to x-ray film for five days and exposed to photographic emulsion for 14 days. All analyses were performed with the observer masked to the animal study group.

Densitometry of autoradiographic images obtained by in situ hybridization was performed by computer-assisted image analysis as previously described [18] according to the methods of Baskin and Stahl [20] using an AIS (Analytical Imaging System, St. Catherine's, Ontario, Canada). In brief, in situ autoradiographic images were placed on a uniformly-illuminating fluorescent light box (Northern Light Precision Luminator Model C60, Image Research, Ontario, Canada) and captured using a digital camera (Fujix HC 2000, Tokyo, Japan) connected to an IBM Pentium III computer with a 1024 \times 1240 pixel array imaging board. Following appropriate calibration by constructing a curve of optical density versus radioactivity [21] quantitation of digitized autoradiographic images was performed using imaging software (AIS). Fifteen non-overlapping 150- μ m fields from the kidney of each rat were quantified as an index of gene expression. Data were expressed as optical density (OD).

In the second approach, changes in expression of EGF and TGF- β within the kidney were explored. Using tissue sections exposed to photographic emulsion for three weeks were examined. Dark-field images were captured and digitized using a Fujix HC-2000 digital camera (Fuji). Regional gene expression was quantitatively measured to determine the proportion of each area occupied by autoradiographic grains as previously described [18], using computerized image analysis (AIS). All sections were hybridized to their respective probes in the same experiment and were analyzed in duplicate [18].

For quantitation of immunohistochemistry, the number of positive tubular cells for either TUNEL or ssDNA was counted in three to five randomly chosen sections from each group ($N = 6$ per group).

Table 1. Body weight, kidney weight, systolic blood pressure and plasma glucose in control, diabetic and diabetic rats treated with perindopril or valsartan

	Body weight g	Kidney weight g	KW/BW $\times 10^{-3}$	Blood pressure mm Hg	Plasma glucose mmol/L
Control	292 ± 8	1.91 ± 0.17	6.5 ± 0.2	209 ± 9	5.4 ± 0.2
Diabetic	221 ± 7 ^a	2.01 ± 0.26	9.1 ± 0.3 ^a	214 ± 11	20.6 ± 1.0 ^b
Perindopril	268 ± 7 ^{ac}	1.89 ± 0.47	7.1 ± 0.6	140 ± 5 ^{ac}	19.5 ± 1.0 ^b
Valsartan	243 ± 12 ^{ac}	1.96 ± 0.24	8.1 ± 0.6	135 ± 4 ^{ac}	21.2 ± 0.7 ^b

Data are expressed as mean ± SEM; $N = 6$ per group. KW/BW, kidney weight/body weight ratio.

^a $P < 0.05$, ^b $P < 0.01$ when compared to control

^c $P < 0.05$ when compared to diabetic

Table 2. Quantitation of epidermal growth factor (EGF) and transforming growth factor- β (TGF- β) mRNA in autoradiographs of kidney cortex and medulla from control, diabetic and diabetic rats treated with perindopril or valsartan

	EGF cortex	TGF- β		
		Medulla	Cortex	Medulla
Control	0.25 ± 0.03	0.29 ± 0.04	0.09 ± 0.03	0.04 ± 0.03
Diabetic	0.15 ± 0.03 ^a	0.31 ± 0.04	0.25 ± 0.04 ^b	0.11 ± 0.04 ^a
Perindopril	0.42 ± 0.03 ^{ad}	0.58 ± 0.05 ^{bd}	0.11 ± 0.02 ^d	0.04 ± 0.02 ^c
Valsartan	0.39 ± 0.03 ^{ad}	0.54 ± 0.04 ^{bd}	0.10 ± 0.06 ^d	0.04 ± 0.02 ^c

Data are expressed as mean ± SEM. EGF and TGF- β are expressed as relative optical density ($N = 6$ per group).

^a $P < 0.05$, ^b $P < 0.01$ when compared to control

^c $P < 0.05$, ^d $P < 0.01$ when compared to diabetic

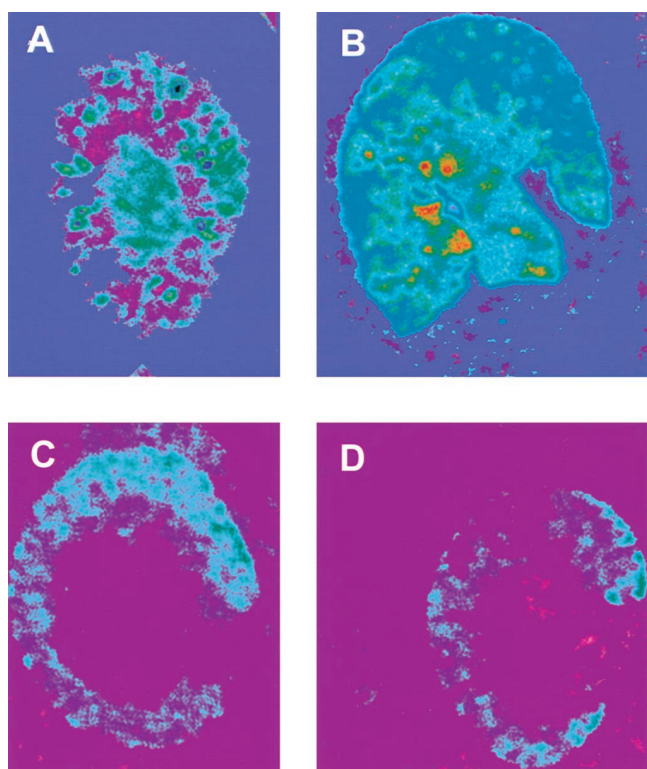


Fig. 1. Low-resolution autoradiographs of transforming growth factor- β (TGF- β) mRNA in the kidney of *Ren-2* rats. (A) Control, (B) diabetic, (C) diabetic treated with perindopril, (D) diabetic treated with valsartan. With diabetes TGF- β is increased when compared to controls while treatment was associated with decreased TGF- β mRNA levels, similar to that of controls. Magnification $\times 5$.

Statistics

Data are expressed as means ± SEM unless otherwise stated. Statistical significance was determined by ANOVA with a Fisher post-hoc comparison. Analyses were performed using Statview II + Graphics package (Abacus Concepts, Berkeley, CA, USA) on an Apple Macintosh G3 computer (Apple Computer, Inc., Cupertino, CA, USA). A P value < 0.05 was regarded as statistically significant.

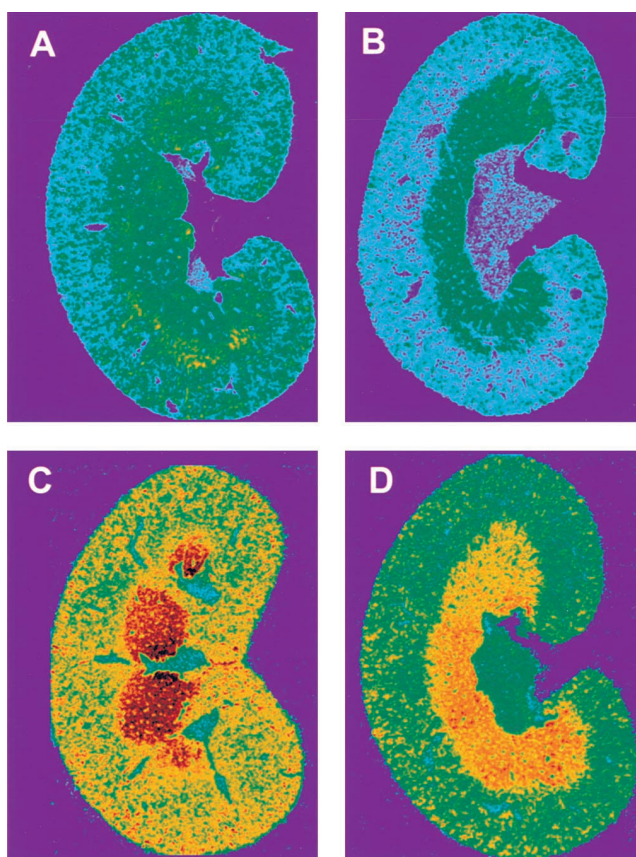


Fig. 2. Low-resolution autoradiographs of growth factor mRNA in the kidney of *Ren-2* rats. (A) Control, (B) diabetic, (C) diabetic treated with perindopril, (D) diabetic treated with valsartan. With diabetes there is reduced EGF mRNA when compared to controls while treatment was associated with increased EGF mRNA to levels above that of controls (magnification $\times 5$).

RESULTS

Rats that received STZ were all diabetic (Table 1). Diabetes was associated with reduced body mass but without change in kidney weight when compared with control animals. Thus, when compared to control animals, the kidney weight/body weight ratio was increased

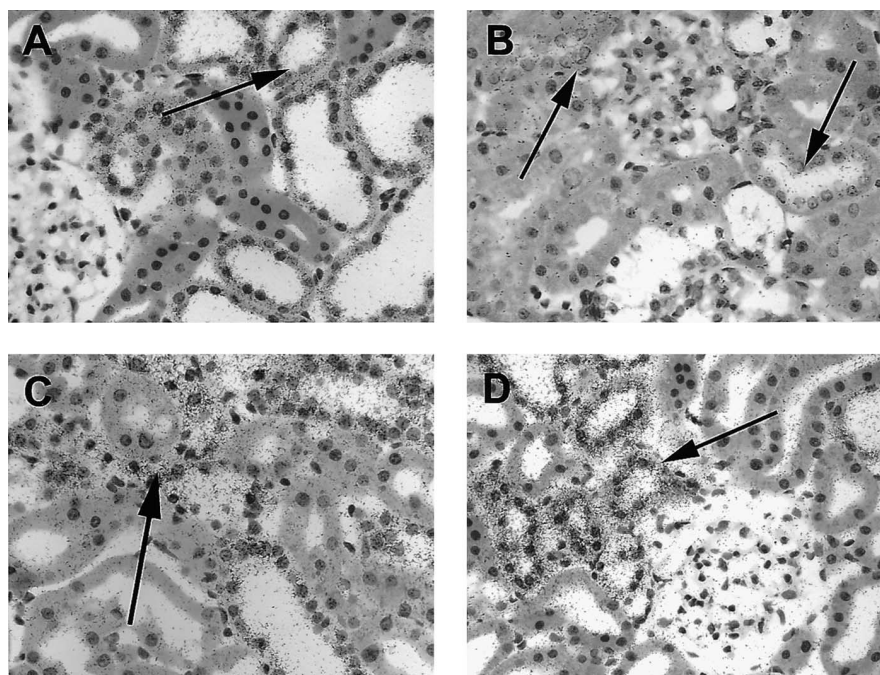


Fig. 3. In situ hybridization of renal EGF mRNA in the *Ren-2* rat. Sections stained with H&E. In control (A) kidneys, EGF mRNA was localized to distal tubules and diabetes (B) was associated with decreased distal tubular EGF gene expression. Both perindopril (C) and valsartan (D) treatment were associated with increased EGF mRNA in distal tubules of diabetic rats (magnification $\times 340$).

Table 3. Quantitation of EGF and TGF- β in situ hybridization in the kidney of control, diabetic and diabetic rats treated with perindopril or valsartan

	EGF	TGF- β
	%	
Control	6.1 \pm 1	0.12 \pm 0.02
Diabetic	3.4 \pm 1.8 ^a	1.24 \pm 0.21 ^a
Perindopril	27.2 \pm 3.5 ^{bc}	0.51 \pm 0.04 ^{ac}
Valsartan	33.2 \pm 2.7 ^{bc}	0.42 \pm 0.06 ^{ac}

Data are expressed as mean \pm SEM. EGF and TGF- β are expressed as % area of grains per tubular area ($N = 6$ per group).

^a $P < 0.05$, ^b $P < 0.01$ when compared to control

^c $P < 0.05$ when compared to diabetic

in diabetic rats (Table 1). Control and diabetic rats were hypertensive, and treatment with both perindopril and valsartan reduced the blood pressure to normotensive levels (Table 1).

Low resolution autoradiography

Compared with control animals, TGF- β mRNA in diabetic rat kidneys was increased, while perindopril and valsartan both attenuated the level of TGF- β gene expression to that similar to controls (Table 2, Fig. 1).

In control animals, EGF mRNA was noted in the cortex and the outer medulla. Compared with control animals, EGF mRNA was decreased in the cortex of diabetic rat kidneys, while perindopril or valsartan treatment increased the level of EGF mRNA in both the cortex and outer medulla to above that of controls (Table 2, Fig. 2).

In situ hybridization microscopy

In control animals, EGF mRNA was localized to the distal convoluted tubules and collecting ducts of the kidney. No EGF mRNA was detected in the glomerulus. Compared with control animals, EGF mRNA in diabetic rat distal tubules (Fig. 3B) was decreased and there was no change in EGF mRNA in the collecting ducts. Treatment with perindopril or valsartan increased the expression of EGF mRNA in both the distal tubules and the collecting ducts to greater than that found in control animals (Table 3, Fig. 3). Sections labeled with sense probe showed no hybridization.

Immunohistochemistry

In control animals, EGF immunolabeling was present in the distal convoluted tubules and collecting ducts of the kidney. No EGF immunolabeling was detected in the glomerulus. Compared with control animals, kidney EGF immunolabeling in diabetic rat distal tubules appeared to decrease. Treatment with perindopril or valsartan was associated with an increase in distal tubular and collecting duct EGF immunolabeling to above that of controls (Fig. 4).

Apoptosis

Tubular apoptosis, as indicated by the immunolabeling for ssDNA monoclonal antibody and TUNEL was increased with diabetes and reduced with either perindopril or valsartan treatment (Table 4, Fig. 5).

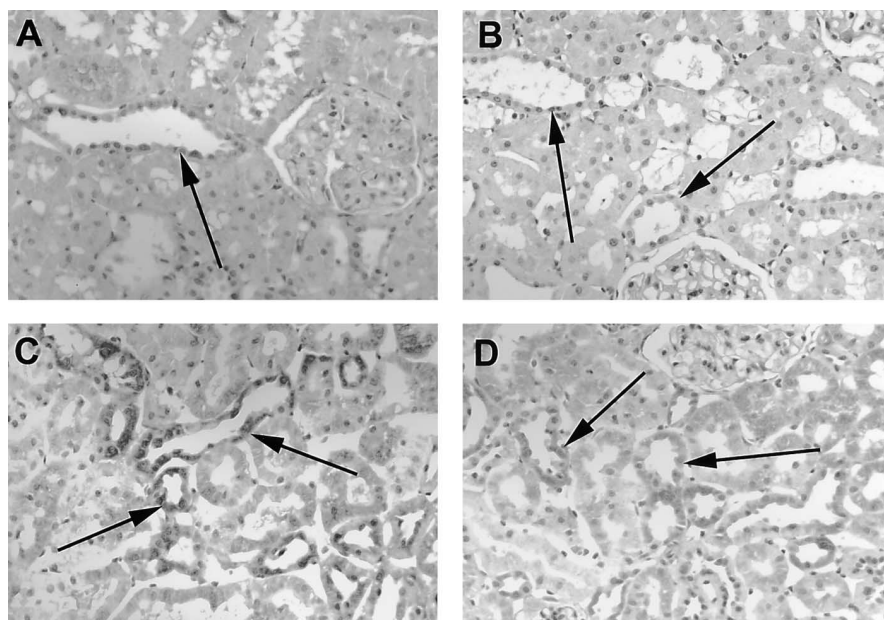


Fig. 4. Immunolabeling for EGF in the kidney of *Ren-2* rats. Sections counterstained with Mayer's hematoxylin. In control (A) kidneys, EGF immunolabeling was localized to distal tubules and diabetes (B) was associated with decreased distal tubular EGF immunolabeling. Both perindopril (C) and valsartan (D) treatment were associated with increased EGF immunolabeling in distal tubules from diabetic rats (magnification $\times 340$).

Table 4. Quantitation of apoptosis and tubular atrophy and fibrosis in control, diabetic and diabetic rats treated with perindopril or valsartan

	TUNEL	ssDNA	Tubular fibrosis	Tubular atrophy
Control	2.4 \pm 1.2	1.2 \pm 0.8	5.1 \pm 1.1	0.5 \pm 0.2
Diabetic	65.3 \pm 11.2 ^b	48.4 \pm 9.7 ^b	19.2 \pm 3.5 ^b	10.2 \pm 1.1 ^b
Perindopril	25.6 \pm 8.5 ^{ac}	16.7 \pm 3.6 ^{ac}	3.8 \pm 0.7 ^{ac}	2.1 \pm 1.0 ^{ac}
Valsartan	19.3 \pm 7.3 ^{ac}	18.2 \pm 3.1 ^{ac}	3.4 \pm 0.9 ^{ac}	2.0 \pm 0.9 ^{ac}

Data expressed as mean \pm SEM. TUNEL and ssDNA are expressed as positive nuclei per field ($N = 6$ per group). Tubule atrophy and fibrosis are expressed as percentage area per 5 tissue sections.

^a $P < 0.05$, ^b $P < 0.01$ when compared to control

^c $P < 0.05$ when compared to diabetics

Tubulointerstitial fibrosis and atrophy

Diabetic rats were associated with significant tubular atrophy and fibrosis when compared to controls (Table 4, Fig. 6). Treatment with either perindopril or valsartan was associated with a significant reduction in both tubular fibrosis and atrophy.

DISCUSSION

The extent of tubulointerstitial injury correlates closely with long-term renal function in a variety of primary glomerular diseases. In particular, tubular atrophy has long been recognized as an indicator of renal disease severity and progression [2, 22]. While such tubular atrophy with its attendant tubular epithelial cell loss may be the result of necrosis, the absence of the attendant cytological features in most chronic renal disease suggests that apoptosis may be the predominant mechanism underlying epithelial cell deletion in the injured kidney.

Indeed, in the present study, not only was renal injury in the diabetic *Ren-2* rat associated with significant tubulointerstitial fibrosis and tubular epithelial cell apoptosis, but renoprotective therapy with RAS blockade ameliorated this mode of cell loss. Furthermore, tubular cells from untreated diabetic *Ren-2* rats expressed an injury-associated growth factor phenotype, with a reduction in EGF expression and a concomitant increase in TGF- β mRNA [5]. These phenotypic changes were reversed by blockade of the RAS with both ACE inhibition and AT1 receptor blockade

The mammalian kidney, particularly the distal tubule, is a major site of constitutive EGF synthesis, where it is thought to act in a paracrine or autocrine manner [23, 24] to modulate a variety of cell functions including cellular metabolism and growth [25]. In the present study, experimental diabetic nephropathy was associated with a reduction in EGF expression suggesting an alteration in the normal tubular phenotype in response to renal injury. These findings are consistent with the reduction in renal EGF reported in both incipient and overt diabetic nephropathy [26] and in tubulointerstitial nephritis in humans [27]. Similarly, reduced renal EGF expression has also been noted in several models of experimental renal disease including chronic allograft rejection [28], renal ischaemia [29] and ureteric obstruction [30]. These models contrast the findings in renal hypertrophy where renal architecture is preserved and EGF expression is transiently increased [31], suggesting that injury rather than growth leads to a reduction in EGF expression. Indeed, activation of the EGF receptor may have a renoprotective phenotype. For instance, the administration of exogenous EGF accelerates the recovery from nephrotoxic

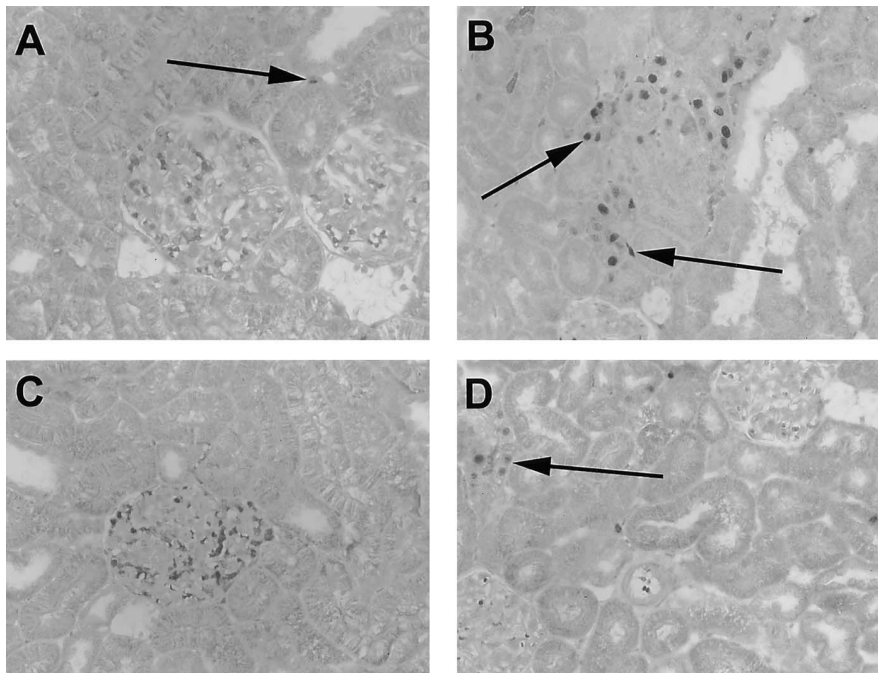


Fig. 5. Immunolabeling for TUNEL in the kidney of *Ren-2* rats. Sections counterstained with eosin. In control rats (A), TUNEL immunolabeling was barely detected while diabetic rats had increased immunolabeling for TUNEL (B). Both perindopril (C) and valsartan (D) treatment were associated with decreased TUNEL positive tubules (magnification $\times 340$).

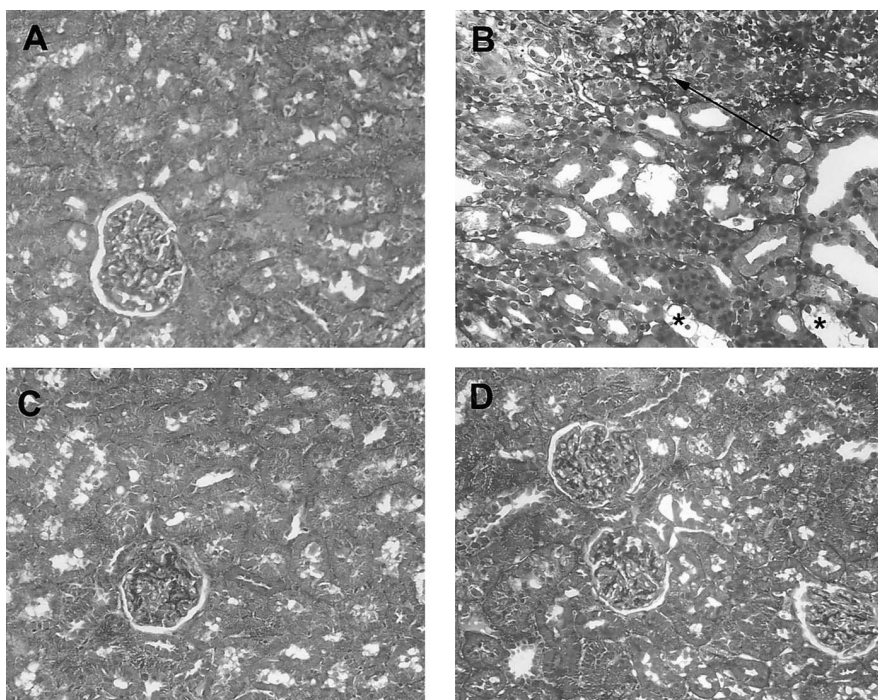


Fig. 6. Masson's trichrome stained section of kidney cortex representing the proportion of tubular atrophy and fibrosis in *Ren-2* rats. In control rats (A), there is very little collagen deposition within the interstitium and no tubular atrophy. However, in the diabetic rats (B) there is significant tubular atrophy (*) and increased tubulointerstitial fibrosis (arrow). Both perindopril (C) and valsartan (D) treatment were associated with decreased tubular atrophy and fibrosis (magnification $\times 340$).

injury [32, 33], ischemia [34], and mechanical obstruction [35]. Furthermore, a related renal growth factor, heparin-binding EGF-like growth factor (HB-EGF), which also signals via the EGF receptor, appears to endogenously exert a renoprotective effect in experimental animals with obstructive uropathy [36]. While the mechanisms mediating this renoprotective effect of EGF have not been

elucidated, recent evidence suggests that activation of the EGF receptor by exogenous EGF [35] or endogenous HB-EGF [36] inhibits tubular epithelial cell apoptosis.

In the present study, blockade of the RAS with both ACE inhibition and AT1 receptor antagonism was associated with up-regulation in EGF expression to supranormal levels in association with reduced apoptosis

and tubular atrophy. These findings suggest that the renoprotective effects of RAS blockade may be mediated, at least in part, by an increase in endogenous EGF.

Transforming growth factor- β is a pleiotropic growth factor with effects on cell proliferation and differentiation as well as its actions on extracellular matrix synthesis and degradation [37]. In addition to cell cycle arrest, TGF- β induces apoptosis in a wide range of cells including glomerular capillary endothelial cells [38], uterine epithelial cells [39] and hepatocytes [40]. However, the pro-apoptotic activity of TGF- β may be altered by the presence of other growth factors. In particular, EGF has been shown to reduce TGF- β induced apoptosis in vitro [9, 41] and in oligonephronal models of chronic renal disease in vivo [3].

The interaction between EGF and the RAS is complex. For instance, EGF is a potent inhibitor of renin secretion [42]. In human diabetic nephropathy [43], as in the diabetic *Ren-2* rat [17] and subtotaly nephrectomized rat [44], renin expression by the tubular epithelium is dramatically increased. These changes are accompanied by increased angiotensin II formation by tubular epithelial cells and overexpression of TGF- β [44, 45]. It is therefore possible that some of the beneficial effects of ACE inhibition and AT1 receptor antagonist, as indicated in the present study, may result from an EGF-mediated reduction in tubular renin expression, with consequent diminution in Ang II-dependent TGF- β synthesis.

In summary, the prominent tubular atrophy and apoptosis noted in the diabetic *Ren-2* rat was associated with reduced EGF and increased TGF- β expression by tubular cells. Blockade of the RAS was associated with attenuation of apoptosis and supranormal expression of EGF. These features suggest that modulation of pro- and anti-apoptotic growth factors may underlie some of the renoprotective effects of blockade of the RAS.

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