Diabetologia (2001) 44: 874–877

Diabetologia

Irbesartan normalises the deficiency in glomerular nephrin expression in a model of diabetes and hypertension

F. Bonnet[1], **M. E. Cooper**[1], **H. Kawachi**[2], **T. J. Allen**[1], **G Boner**[3], **Z. Cao**[1]

[1] Department of Medicine, University of Melbourne, Austin and Repatriation Medical Centre, Heidelberg West, Victoria, Australia
[2] Department of Cell Biology, Institute of Nephrology, Niigata University School of Medicine, Niigata, Japan
[3] Institute of Hypertension and Kidney diseases, Rabin Medical Center, Tel Aviv University, Tel Aviv, Israel

Abstract

Aims/hypothesis. The location of nephrin has been identified as the slit-diaphragm of the glomerular podocyte. Recent evidence suggests that nephrin could play a key role in the function of the glomerular filtration barrier and the development of proteinuria but its status in long-term diabetes is still not understood. We studied the expression of nephrin in a hypertensive model of diabetic nephropathy and investigated the potential influence of angiotensin II blockade on nephrin gene and protein expression.
Methods. Streptozotocin-diabetic spontaneously hypertensive rats were given either no treatment or the angiotensin II antagonist, irbesartan, at a dose of 15 mg/kg per day by gavage for 32 weeks. Non-diabetic spontaneously hypertensive rats were used as a control group. Real time RT-PCR and immunohistochemistry were used to assess and quantify gene and protein expression of nephrin.
Results. Diabetic spontaneously hypertensive rats developed albuminuria and had a reduction in both gene and protein expression of nephrin when compared with control rats. Irbesartan treatment prevented the development of albuminuria and completely abrogated the down regulation of nephrin in diabetic rats.
Conclusion/interpretation. Long-term diabetes in spontaneously hypertensive rats is associated with a reduction in both gene and protein expression of nephrin within the kidney. These changes in nephrin levels were completely prevented by angiotensin II antagonist treatment, suggesting a potential novel mechanism to explain the antiproteinuric effect of agents which interrupt the renin-angiotensin system. [Diabetologia (2001) 44: 874–877]

Keywords Nephrin, diabetes, angiotensin II, angiotensin II receptor antagonist, diabetic nephropathy, irbesartan, SHR.

Diabetic nephropathy is a leading cause of the development of end-stage renal disease in the Western world. Recent studies have shown that the human gene NPHS1 protein product, nephrin, is located in the slit diaphragm of the glomerular podocyte barrier [1, 2]. The massive proteinuria observed in patients with mutation of the NPHS1 gene suggests a key role for nephrin in the function of the glomerular filtration barrier. Furthermore, different groups have consistently shown a reduction in nephrin expression in experimental glomerular injury and in acquired human proteinuric renal diseases, suggesting that nephrin could contribute to the pathogenesis of proteinuric nephropathies [3, 4]. However, the expression of nephrin in overt diabetic nephropathy is not well understood.

A large body of clinical and experimental studies has demonstrated that the blockade of the renin angiotensin system using angiotensin II receptor antagonists reduces proteinuria and delays the progression

Received: 19 January 2001 and in revised form: 16 March 2001

Corresponding author: M. Cooper, Dept. of Medicine, University of Melbourne, Austin and Repatriation Medical Centre, Heidelberg West, Victoria 3081, Australia

Abbreviations: SHR, spontaneously hypertensive rat; RAS, renin-angiotensin-system; SBP, systolic blood pressure; AER, albumin excretion rate; AT1 receptor, angiotensin type 1 receptor; RT-PCR, reverse transcription-polymerase chain reaction.

of renal injury associated with diabetes [5]. Nevertheless, the regulation of expression of nephrin following angiotensin II blockade in diabetes has not yet been determined. The aim of our study was to investigate the level of expression of both gene and protein nephrin in a hypertensive model of diabetic nephropathy, the streptozotocin-diabetic spontaneously hypertensive rat (SHR) [6]. We selected this model because it exhibits diabetes-associated increased albuminuria and increased glomerular ultrastructural injury [6]. Furthermore, hypertension is commonly associated with overt diabetic nephropathy and plays a pivotal role in its prognosis. We also explored whether an antiproteinuric agent such as the angiotensin II antagonist, irbesartan, has concomitant effects on nephrin expression.

Material and methods

We used 8-week old male spontaneously hypertensive rats (SHR, body weight 230–260 g) housed at the Biological Research Laboratory in the Austin and Repatriation Medical Centre in this study. The research protocol was approved by the Animal Welfare Committee of Austin and Repatriation Medical Centre. A total of 30 rats were randomly allocated to receive streptozotocin (Boehringer-Mannheim, Mannheim, Germany) at a dose of 45 mg/kg in citrate buffer after a 16-h fast (diabetic, $n = 20$) or to receive citrate buffer alone (control, $n = 10$) [6]. Long-acting insulin (Ultralente, Novo Industries, Copenhagen, Denmark) at a dose of 4 U/day was given to all diabetic rats by subcutaneous injection to avoid ketonuria. The rats had unrestricted access to water and standard rat chow. Following the induction of diabetes, the animals were further randomized to receive either no treatment ($n = 10$) or the AT1 receptor antagonist irbesartan (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, N.J., USA) at a dose of 15 mg/kg body weight/day by gavage once daily for 32 weeks ($n = 10$).

Systolic blood pressure (SBP) was assessed by tail cuff plethysmography in conscious preheated rats every 4 weeks. Before being killed, all rats were placed in metabolic cages (Iffa Credo, L'Arbresele, France) to collect urine over 24 h for measurement of albumin concentration by radioimmunoassay as previously described [6]. Before being killed, blood from the rats was collected for determination of HbA_{1c} by HPLC (Biorad, Richmond, Calif., USA).

At week 32, rats were anaesthetized by intravenous injection of pentobarbitone sodium (60 mg/kg body weight, Boehringer Ingelheim, Artarmon, NSW, Australia). Both kidneys were removed, bisected and snap frozen in liquid nitrogen-cooled isopentane and stored at –20 °C.

Immunohistochemistry. Immunohistochemical staining of nephrin was performed as previously described according to a modified method using a specific antibody to mAb 5–1–6 antigen which is identical to rat nephrin [2]. This antibody was raised in the rabbit against a synthetic peptide sequence derived from the cytoplasmic region of the nephrin protein [2]. In brief, 6 micron 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/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 multi-link swine anti-goat, mouse, rabbit immunoglobulin (DAKO, Copenhagen, Denmark) 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, Miss., USA) substrate. Quantification of nephrin immunostaining was done by the calculation of the proportion of area occupied by the brown staining within each glomerulus [7]. An observer who was masked to the study group of origin assessed 15 glomeruli for each slide and 8 slides for each rat.

Reverse transcription-polymerase chain reaction. Three micrograms of total RNA extracted from each kidney was used to synthesize cDNA with the Superscript First Strand synthesis system for RT-PCR (Gibco BRL, Grand Island, N.Y., USA). Nephrin mRNA expression was analysed by real-time quantitative RT-PCR using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer, Foster City, Calif., USA). Fluorescence for each cycle was quantitatively analysed by an ABI Prism 7700 Sequence Detection System (Perkin-Elmer, PE Biosystems, Foster City, Calif., USA). To control for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalised in relation to the expression of an endogenous control, 18S ribosomal RNA (rRNA) (18S rRNA TaqMan Control Reagent kit; ABI Prism 7700, Perkin-Elmer, Foster City, Calif., USA). Primers and Taqman probe for nephrin and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700, Perkin-Elmer, Foster City, Calif., USA). For nephrin, the forward primer was 5' TGGCCATCGGAGGCAA 3' and the reverse primer was 5' CGCTCACCGGACGTGAA 3'. The probe specific to nephrin was FAM-5' -CCAGACCCCTCCCTCATCTGGTTTAAGG-3'-TAMRA; FAM = 6 carboxyfluorescein, TAMRA (quencher) = 6-carboxy-tetramethylrhodamine. The amplification was performed with the following time course: 50 °C, 2 min and 10 min at 95 °C; and 40 cycles of 94 °C, 20 sec, 60 °C, 1 min. Each sample was tested in triplicate. Results were expressed as relative to control kidneys, which were arbitrarily assigned a value of 1.

Statistical Analysis. Data were analysed by analysis of variance (ANOVA) using Statview SE (Brainpower, Calabasas, Calif., USA). Comparisons of group means were performed by Fisher's least significant difference method. Because albuminuria did not have a normal distribution, this parameter was analysed after logarithmic transformation. Data are shown as means ± SEM, unless otherwise specified. A p value of less than 0.05 was viewed as statistically significant.

Results

Animal data. At the end of the experiment, diabetic SHRs had an increased HbA_{1c} value compared to non-diabetic SHR rats (non-diabetic, 5.0 ± 0.3 % vs diabetic, 7.9 ± 0.6 %, $p < 0.01$). Glycaemic control was not influenced by irbesartan treatment (7.8 ± 0.8 %). The mean systolic blood pressure

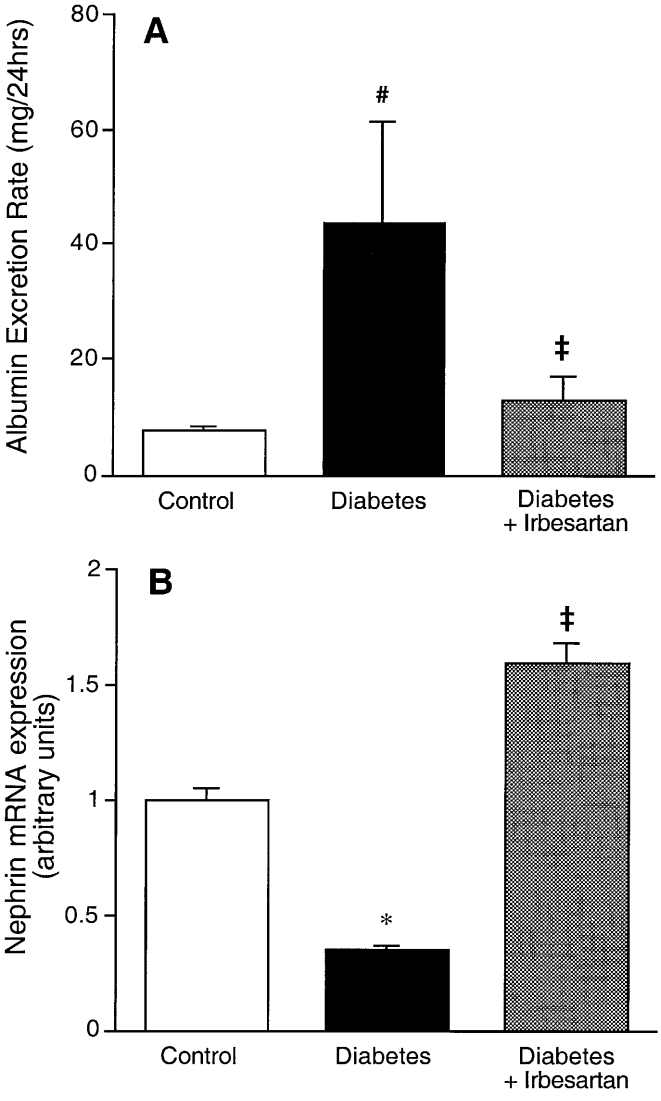

Fig. 1. Albumin excretion rate (**A**) and kidney nephrin gene expression (**B**) in control, diabetic, and diabetic SHR rats treated with irbesartan. Albuminuria is shown as geometric mean × /÷ tolerance factor. Nephrin gene expression is expressed relative to control level. *$p < 0.05$ vs control; #$p < 0.01$ vs control; ‡$p < 0.01$ vs diabetes

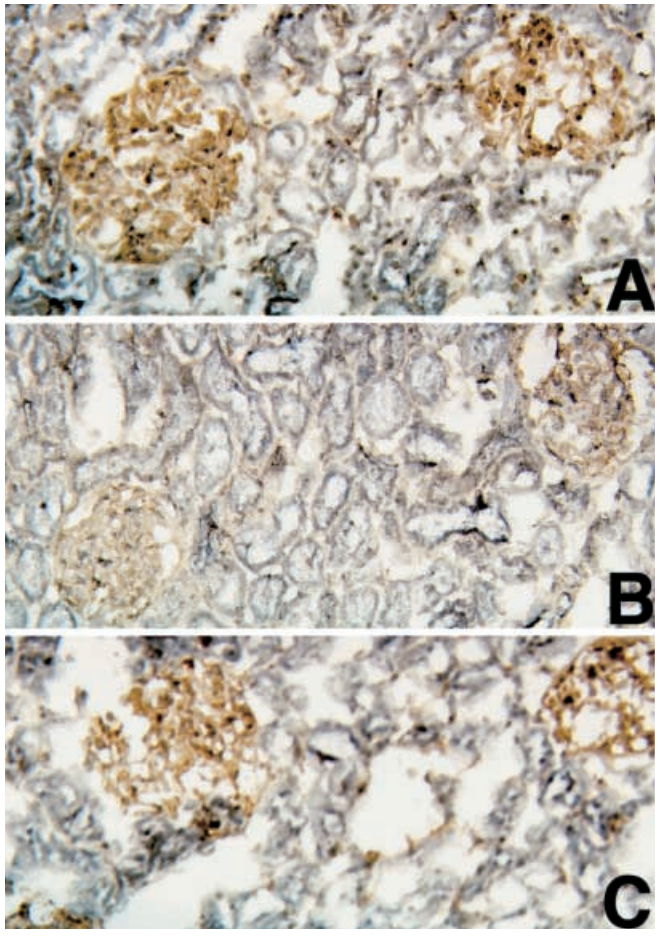

Fig. 2. Immunohistochemical staining for nephrin in kidney from control, (**A**) diabetic, (**B**) and diabetic SHR treated with irbesartan. (**C**) Positive staining is shown as brown. Sections are counterstained with haematoxylin. Original magnification × 200

(SBP) over the 32 weeks of experimental period was modestly reduced in untreated diabetic SHRs (204 ± 4 mmHg) compared with the control animals (225 ± 3 mmHg, $p < 0.05$ vs control). Treatment with the AT1 receptor antagonist irbesartan was associated with a reduction in SBP compared to the untreated diabetic rats (168 ± 4 mmHg, $p < 0.01$).

Albumin excretion rate (AER) was significantly increased in diabetic SHRs when compared to non-diabetic SHRs. Irbesartan treatment in the diabetic SHRs prevented the increase in albuminuria with concentrations similar to that in control animals (Fig. 1A).

Gene expression of nephrin. Diabetic SHRs had a significant reduction in nephrin gene expression when compared to control SHRs (Fig. 1B). Irbesartan treatment was associated with a significant increase in nephrin mRNA when compared to untreated diabetic rats (Fig. 1B). The level of nephrin mRNA in irbesartan-treated diabetic animals was not significantly different to the level observed in untreated diabetic rats.

Immunohistochemistry for nephrin. Diabetic SHRs had a reduction in glomerular immunostaining for nephrin when compared with control rats (glomerular nephrin protein content: control, 10.1 ± 0.7 vs diabetic, 6.3 ± 1.1 %, $p < 0.05$; Fig. 2). Irbesartan treatment was associated with an increase in the immunostaining for nephrin when compared with untreated diabetic animals (12.0 ± 1.6 %, $p < 0.05$ vs diabetic; Fig. 2).

Discussion

This study shows that nephrin expression is significantly reduced in long-term diabetic SHRs and that treatment with an angiotensin II receptor antagonist com-

pletely blunted these changes in nephrin expression and also prevented the development of proteinuria.

Nephrin is a transmembrane cell adhesion protein that contains multiple immunoglobulin-like motifs in its extra-cellular domain. Mutations in the nephrin gene, NPHS1, have been shown to lead to a severe congenital nephrotic syndrome [1]. Recently, nephrin protein has been located to the slit diaphragm of glomerular podocytes, suggesting a role for nephrin in the renal ultrafilter barrier function [1, 2]. This study detected a down regulation of the nephrin gene and protein expression in a model combining diabetes and hypertension. Our findings are consistent with recent studies that have demonstrated a reduction in nephrin expression after induction of different experimental proteinuric diseases [3]. Furthermore, recent reports in humans have also shown decreased nephrin expression in patients with proteinuria secondary to acquired non-diabetic glomerular diseases [4]. This study expands the association of nephrin with proteinuria from the Finnish type of congenital nephrotic syndrome and other experimental models of non-diabetic renal diseases to diabetic nephropathy.

It has been suggested that filtration slit structure injury and, in particular, podocyte loss contribute to the progressive development of glomerulosclerosis and albuminuria in diabetic nephropathy. A study in diabetic Pima Indians has shown that patients with overt nephropathy and proteinuria had fewer podocytes per glomerulus than subjects with less advanced nephropathy [8].

The mechanisms leading to the down regulation of nephrin in glomerular injury including diabetic nephropathy are still not well understood. The potential role of reactive oxidants and, particularly, the balance of lipid peroxidation has been recently proposed to account for the drop of nephrin mRNA in experimental diseases [3]. Further studies are required to confirm the relevance of this phenomenon to diabetic nephropathy. This study cannot determine if the decrease in nephrin expression is the cause of proteinuria or the effect of advanced renal injury, particularly podocyte injury. It has been speculated that proteinuria is mediated at least in part by the disruption of the slit-diaphragm at the molecular level. However, one cannot exclude that diabetes-induced podocyte injury could contribute to the decrease in nephrin expression. The influence of increased blood pressure itself on nephrin expression appears less prominent because, in this study, diabetic SHRs had a lower SBP but also lower nephrin expression than control rats, suggesting that the observed decrease also involves glucose-dependent pathways.

The links between the RAS and the regulation of nephrin expression have not been clarified. Renoprotection conferred by the blockade of the RAS has been shown to be associated with the preservation of slit diaphragm function and glomerular distribution of protein zonula occludens-1 (ZO-1), a component of the podocyte slit-diaphragm [9]. A preliminary report has also shown that angiotensin II antagonist could prevent the down regulation of nephrin gene expression in a model of progressive non-diabetic renal injury [10]. These findings are consistent with those of our study that demonstrate that angiotensin II antagonist treatment completely blunts the drop in nephrin expression in diabetic SHRs. We found the influence of angiotensin II receptor antagonist on nephrin expression to be associated with a concomitant reduction in proteinuria, suggesting that at least part of the renoprotective effects of this treatment could be related to a specific action on the renal ultrafilter barrier.

It remains to be determined if the influence of the angiotensin II antagonist on nephrin expression relates to blood pressure reduction, intra-glomerular haemodynamic changes or to blocking the local actions of angiotensin II. In summary, this study shows a significant reduction in both gene and protein expression of nephrin in long-term diabetic SHRs with proteinuria. These changes in nephrin levels were completely prevented by angiotensin II antagonist treatment, suggesting another mechanism of action for this anti-proteinuric class of agents.

Acknowledgements. F. Bonnet was supported by a grant from the French Society of Endocrinology and Ipsen-Biotech. This research was supported by a Centre grant from the Juvenile Diabetes Foundation International and the National Health and Medical Research Council of Australia.

References

1. Ruotsalainen V, Ljungberg P, Wartiovaara J et al. (1999) Nephrin is specifically located at the slit diaphragm of glomular podocytes. Proc Natl Acad Sci USA 96: 7962–7967
2. Kawachi H, Koike H, Kurihara H et al. (2000) Cloning of rat nephrin: expression in developing glomeruli and in proteinuric states. Kidney Int 57: 1949–1961
3. Luimula P, Ahola H, Wang SX et al. (2000) Nephrin in experimental glomerular disease. Kidney Int 58: 1461–1468
4. Furness PN, Hall LL, Shaw JA, Pringle JH (1999) Glomerular expression of nephrin is decreased in acquired human nephrotic syndrome. Nephrol Dial Transplant 14: 1234–1237
5. Allen TJ, Cao Z, Youssef S, Hulthen UL, Cooper ME (1997) Role of angiotensin II and bradykinin in experimental diabetic nephropathy. Functional and structural studies. Diabetes 46: 1612–1618
6. Cooper ME, Allen TJ, O'Brien RC et al. (1988) Effects of genetic hypertension on diabetic nephropathy in the rat: functional and structural characteristics. J Hypertens 6: 1009–1016
7. SoulisT, Thallas V, Youssef S et al. (1997) Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. Diabetologia 40: 619–628
8. Pagtalunan ME, Miller PL, Jumping-Eagle S et al. (1997) Podocyte loss and progressive glomerular injury in type II diabetes. J Clin Invest 99: 342–348
9. Macconi D, Ghilardi M, Bonassi ME et al. (2000) Effect of angiotensin-converting enzyme inhibition on glomerular basement membrane permeability and distribution of zonula occludens-1 in MWF rats. J Am Soc Nephrol 11: 477–489
10. Benigni A, Tomasoni S, Gagliardini E et al. (2001) Blocking angiotensin II synthesis/activity preserves glomerular nephrin in rats with severe nephrosis. J Am Soc Nephrol 12: 941–948