Expression of the slit-diaphragm protein, nephrin, in experimental diabetic nephropathy: differing effects of anti-proteinuric therapies

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Abstract

Background. Mutations in the gene coding for the podocyte slit-pore membrane protein, nephrin, are responsible for the Finnish-type congenital nephrotic syndrome. The present study sought to examine whether nephrin expression may also be altered in experimental diabetes, and how such changes related to the development of proteinuria. In addition, the study also sought to examine nephrin expression in animals treated with different anti-proteinuric therapies.

Methods. Nephrin gene expression and localization were examined in rats with streptozotocin-induced diabetes at 6 months duration (proteinuric phase) and at 7 days (pre-proteinuric phase). In addition, the effects of anti-proteinuric drug therapies were also assessed in long-term diabetic rats, treated with either the angiotensin-converting enzyme inhibitor perindopril, or the blocker of advanced glycation end-product formation, aminoguanidine. Nephrin expression was determined using quantitative real-time PCR and in situ hybridization.

Results. When compared with control animals, nephrin expression was reduced in the late proteinuric phase (45% reduction vs controls, P < 0.01) but not in the early, pre-proteinuric phase of experimental diabetic nephropathy. While ACE inhibition and aminoguanidine both reduced proteinuria, only the former attenuated the diabetes-associated reduction in nephrin expression.

Conclusions. These findings suggests that reduction in nephrin may be a determinant of glomerular hyperpermeability in diabetic nephropathy. Attenuation of these changes with ACE inhibition suggest that this mechanism may contribute to the anti-proteinuric effects of this, but not all classes of drug which reduce urinary protein in diabetic nephropathy.

Keywords: advanced glycation end products; angiotensin II; diabetes; nephrin; podocyte; proteinuria

Introduction

Diabetic nephropathy, like many glomerulopathies, is characterized by the early development of proteinuria followed by a later decline in glomerular filtration in association with glomerulosclerosis. However, while considerable advances have been made in unravelling the pathogenesis of glomerulosclerosis in diabetes [1], the mechanisms underlying the development of proteinuria in diabetic kidney disease remain less well understood. Changes in the structure of the glomerular basement membrane (GBM) and its anionic sites have been postulated to affect the transglomerular passage of albumin in diabetes, as have changes in growth factors acting directly on endothelial cells [2]. However, the podocyte slit-membrane has also been implicated in the pathophysiology of proteinuria with the discovery of nephrin, mutations that lead to congenital nephrotic syndrome, NPHS1 [3]. More recently, changes in nephrin expression have also been implicated in acquired proteinuric disease [4].

Agents that block the renin–angiotensin system (RAS), such as angiotensin-converting enzyme (ACE) inhibitors, reduce proteinuria to a greater extent than would be expected by blood pressure reduction alone. Indeed, the reduction in proteinuria by RAS blockade may also account for the effectiveness of this strategy in reducing the attendant decline in glomerular filtration rate (GFR) [5], with proteinuria strongly implicated in the pathogenesis of secondary tubulo-interstitial disease [6]. However, while there has been
a presumption that the anti-proteinuric effects of ACE inhibitors reflect changes in intraglomerular haemodynamics, the podocyte may also contribute to the effectiveness of this class of drug.

Long-lived non-enzymatically glycated proteins referred to as advanced glycation end-products (AGEs) have also been implicated in the pathogenesis of diabetic nephropathy. For instance, the administration of AGEs to non-diabetic animals leads to albuminuria [7], and inhibition of advanced glycation with aminoguanidine reduces proteinuria in experimental diabetes [8]. In addition, these findings raise the possibility that AGEs may also induce proteinuria by alterations in podocyte structure and function.

The aims of the present study were twofold. Firstly, we sought to examine whether nephrin gene expression was altered in experimental diabetes before or after the development of proteinuria. Secondly, the study sought to examine the effects on nephrin expression of two different anti-proteinuric therapies: ACE inhibition and aminoguanidine.

Subjects and methods

Animals

Experimental studies were conducted in two stages. Stage 1 was conducted to examine changes in glomerular nephrin gene expression early in the course of experimental diabetes, following significant renal hypertrophy but prior to the development of proteinuria. The aims of stage 2 were twofold. Firstly, to examine nephrin expression in long-term, proteinuric diabetes. Secondly, to determine the effects of anti-proteinuric treatment with ACE inhibition and inhibition of AGE formation with aminoguanidine.

In study 1, 14 male Sprague–Dawley rats aged 13 weeks were randomly assigned to control and diabetic groups. Diabetes was induced in seven rats by the intravenous administration of streptozotocin (STZ) 45 mg/kg body weight. Rats were given ad libitum access to water and standard chow containing 20% protein (Clark, King & Co, Melbourne, Australia). Only STZ-treated animals with plasma glucose levels >15 mmol/l were considered diabetic and included in the study. Animals were sacrificed at 7 days following STZ injection. At sacrifice, the left kidney was decapsulated and glomeruli isolated by serial sieving as previously described [12]. In brief, a 293 base pair cDNA coding for nephrin was amplified and cloned into the pGEM-T (Promega) vector as previously described [12]. In brief, a 293 base pair cDNA coding for nephrin was amplified and cloned into the pGEM-T (Promega) vector as previously described [12].

Quantitative real-time RT-PCR

Nephrin gene expression was quantified by real-time RT-PCR using sequence specific primers as previously reported by our group, with forward primer (5’ to 3’): TAATGTGTCTCTGGCCACCA, reverse primer (5’ to 3’): TTGGTGTTGGTTCAAGGCAAG, and probe: FAM-CCCTCCTCAAGCAGCAGCCACCA-TAMRA [11]. A commercial, pre-developed 18S control kit labelled with the fluorescent reporter dye (VIC) on the 5’ end and the quencher (TAMRA) on the 3’ end (PE Biosystems, Foster City, CA, USA) was used as the housekeeping gene to control for inequalities of loading. Primers and probes for target genes were obtained from PE Biosystems. The probe for nephrin included a fluorescence reporter (6-carboxyfluorescein (FAM)) at the 5’-end and a fluorescent quencher (6-carboxytetramethylrhodamine (TAMRA)) on the 3’-end. For the relative quantification of the target gene and the endogenous reference 18S ribosomal RNA (18S), real-time quantitative RT-PCR was performed using a GeneAmp 5700 Sequence Detector (PE Biosystems) according to the manufacturer’s instructions. The derived normalized values are the averages of four runs.

In situ hybridization

An anti-sense riboprobe was generated as previously described [12]. In brief, a 293 base pair cDNA coding for rat nephrin was cloned into pGEM-T (Promega), linearized with Not I and an anti-sense riboprobe was produced using T7 RNA polymerase. Purified riboprobe length was adjusted to approximately 150 bases by alkaline hydrolysis.
Four-μm-thick sections were cut onto slides precoated with 3-aminopropyltriethoxysilane and baked overnight at 37°C. Tissue sections were dewaxed and rehydrated, and in situ hybridization was performed using radiolabelled riboprobe as previously described [12]. Following hybridization, slides were washed in 2× SSC for 45 min at 55°C, dehydrated in graded ethanol, air dried, and exposed to Kodak X-Omat autoradiographic film for 3 days. Slides were then dipped in Ilford K5 nuclear emulsion (Ilford, Mobberley, Cheshire, UK), stored in a light-free box with desiccant at 4°C for 21 days, immersed in Kodak D19 developer, fixed in Ilford Hypan, and stained with haematoxylin and eosin.

Statistics

Because of its highly skewed distribution, albumin excretion rate (AER) was logarithmically transformed before statistical analysis and expressed as the geometric mean ×/× tolerance factor. Between group differences were analysed by ANOVA with correction for multiple comparisons using the Fisher’s least significant difference test. Linear regression analysis was used to determine the correlation between the magnitude of nephrin gene expression and proteinuria. Analyses were performed using the Statview SE+ Graphics package (Abacus Concepts, Calabasas, CA, USA) on an Apple Macintosh G4 (Apple Computer Inc, Cupertino, CA, USA). A P value <0.05 was considered statistically significant.

Results

Animal characteristics

Rats that had received STZ were all diabetic (blood glucose >15 mmol/l). Animals with short-term diabetes (study 1) had renal enlargement when expressed either as kidney weight or kidney : body weight ratio (Table 1). Long-term diabetic animals were similarly hyperglycaemic and had increased urinary albumin excretion compared with control animals (Table 2). Perindopril treatment was associated with a reduction in blood pressure and albuminuria but did not influence glycaemic control. Aminoguanidine treatment was accompanied by a reduction in albuminuria but without changes in either blood pressure or glycaemic control. Diabetes was associated with lower body weight that was unaffected by treatment with either perindopril or aminoguanidine (Table 1).

Real time and RT-PCR

In study 1, short-term diabetes was not associated with altered nephrin gene expression (Table 1). However, in study 2, glomerular nephrin gene expression, as assessed by quantitative real time RT-PCR, was reduced in long-term diabetic rats compared with control animals (Figure 1). Nephrin mRNA in perindopril-treated diabetic rats was greater than that of untreated diabetic animals and similar to that in glomeruli from non-diabetic controls. In contrast, in aminoguanidine-treated rats, nephrin expression was similar to untreated diabetic animals. No correlation was noted between nephrin expression and AER.

In situ hybridization

Emulsion-dipped, haematoxylin and eosin counterstained sections localized nephrin mRNA exclusively to the glomerulus. Nephrin mRNA was detected in a predominantly peripheral distribution consistent with expression in visceral epithelial cells in both control and diabetic rat kidneys (Figure 2). Fewer autoradiographic grains were detected in glomeruli of diabetic rats and diabetic animals treated with aminoguanidine. In perindopril-treated diabetic rats, nephrin gene expression was similar to control animals. There was no evidence of extraglomerular expression in animals from either group. No hybridization

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Table 2. Clinical characteristics of rats in study 2

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + perindopril</th>
<th>Diabetic + aminoguanidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>615±14</td>
<td>384±15*</td>
<td>401±11*</td>
<td>390±15*</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.4±0.1</td>
<td>1.7±0.1*</td>
<td>1.9±0.1*</td>
<td>1.8±0.1*</td>
</tr>
<tr>
<td>Haemoglobin A1c (%)</td>
<td>3.1±0.1</td>
<td>10.4±0.4*</td>
<td>10.4±0.7*</td>
<td>10.1±0.3*</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>100±3</td>
<td>138±9*</td>
<td>103±6*</td>
<td>140±10*</td>
</tr>
<tr>
<td>Albuminuria (mg/day)</td>
<td>0.6±/×1.2</td>
<td>10.8±/×1.4*</td>
<td>2.9±/×1.2*</td>
<td>3.8±/×1.5*</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM except for albuminuria where geometric means ×/× tolerance factors are shown. *P<0.01 vs control, †P<0.05 vs diabetic.
Glomerular nephrin mRNA in experimental diabetic nephropathy. mRNA were quantified using real-time RT-PCR in glomeruli from control and diabetic rats receiving perindopril, aminoguanidine, or no treatment. Results are expressed as threshold cycle (Ct) for nephrin standardized to Ct for the housekeeping gene 18S. Values are means ± SEM relative to controls, which were arbitrarily assigned a value of 1. *P < 0.01 vs control, |P < 0.01 vs diabetic.

Discussion

Since the cloning of nephrin in 1998 [13] and the demonstration its crucial role in the development and function of the glomerular filtration barrier [3], investigation into its potential role in the pathogenesis of acquired proteinuric diseases has been the subject of intensive research. The present study demonstrates several findings in relation to nephrin in diabetic nephropathy. Firstly, nephrin expression is reduced in the late proteinuric phase, but not in the pre-proteinuric phase of experimental diabetic nephropathy, suggesting an association between nephrin and proteinuria rather than hyperglycaemia per se. Secondly, while both ACE inhibition and aminoguanidine reduce proteinuria, only the former attenuated the diabetes-associated reduction in nephrin expression, suggesting that different cell-specific interactions may account for the anti-proteinuric effects of these drugs.

The normal glomerulus rigorously restricts the transcapillary passage of large-molecular-weight proteins whilst permitting the permeation of smaller molecules, electrolytes, and water. Disruption of this barrier function results in excessive transcapillary passage of protein and proteinuria. The complexities of the glomerular barrier are only beginning to be unravelled, with recent studies suggesting a key role for the podocyte slit-pore protein, nephrin [3], mutations of which are responsible for the Finnish-type congenital nephrotic syndrome [3]. More recently, the role of nephrin in the pathogenesis of acquired proteinuric disease has also been explored, with variable changes in expression documented in a range of human glomerulopathies [4] and experimental models [14]. In the present study, nephrin expression was reduced in glomeruli of rats with long-term, proteinuric diabetes but not in animals with short-term diabetes, studied prior to the development of proteinuria. These findings suggest that the reduction in nephrin expression is not the result of hyperglycaemia per se.

As in humans, renal hypertrophy is also a characteristic feature of experimental diabetes. The glomerulus in particular undergoes a rapid increase in size, such that glomerular volume increases by 30% of experimental diabetes [15]. However, in the present study, nephrin expression during this early phase of diabetic glomerulopathy remained unchanged, suggesting that slit-pore composition remained constant and its barrier function is unperturbed by hyperglycaemia per se. In contrast, in long-term, proteinuric, diabetic rats, nephrin gene expression was significantly reduced. This reduction in nephrin expression may be either a primary or a secondary phenomenon. For instance, it is possible that nephrin expression may be reduced as a consequence of podocyte loss, as documented in micro- and macroalbuminuric diabetic subjects, but not those with normoalbuminuria [16]. However, it is also possible that a primary reduction in nephrin may destabilize and consequently disrupt the slit-membrane structure, thereby leading to podocyte loss and proteinuria.

In addition to reducing proteinuria, in the present study, ACE inhibitor treatment was associated with normalization of nephrin gene expression. ACE inhibitors have long been recognized as potent antiproteinuric agents, reducing urinary protein excretion beyond that expected due to blood pressure reduction alone. Indeed, the anti-proteinuric effect of this class of drugs is viewed as an important factor in their renoprotective effects in both diabetic and non-diabetic kidney diseases [5,6]. However, the mechanisms underlying the anti-proteinuric effects of blockade of the RAS are not completely understood. Angiotensin II is known to regulate glomerular function by modulation of arteriolar tone and ultrafiltration co-efficient Kf [17]. More recently, functioning receptors for angiotensin II have been identified in the podocyte in freshly isolated intact glomeruli [18]. Furthermore, in contrast to other classes of anti-hypertensive agents, blockade of the RAS preserves podocyte structure following renal mass reduction [19], suggesting a direct effect of angiotensin II on podocyte integrity. Similarly, in experimental diabetic nephrophyathy, both ACE inhibitor and angiotensin-receptor blocker attenuated the accompanying decrease in the number of podocyte slit pores per unit length of GBM [20]. Together with the present study, these findings suggest that the renoprotective effects of blockade of the RAS may include preservation of podocyte number, structure, and molecular composition, with nephrin a key component of this structure–function relationship [3]. However, in addition to blockade of
the RAS, it is possible that the hypotensive effects of perindopril, as demonstrated in the present study, will have also contributed to the described changes, by reducing podocyte barotrauma.

As a consequence of increased substrate (glucose) availability, AGEs accumulate at an accelerated rate in patients with diabetes where they have been postulated to play a major role in the pathogenesis of diabetic nephropathy. Inhibition of AGE formation with aminoguanidine, reduces proteinuria in diabetic animals [8], although the mechanisms underlying this effect are not well understood and while this agent has been shown to reduce fractional mesangial volume [8], in contrast to ACE inhibition, it does not affect diabetes-induced GBM thickening [8]. These findings, in conjunction with those of the present study in which aminoguanidine did not attenuate the diabetes-associated reduction in nephrin expression, suggest that the anti-proteinuric effects of this agent may be mediated by non-podocyte-related mechanisms such as modulation of lysosomal processing [21]. However, whether aminoguanidine might also affect podocyte number and morphological parameters such as foot-process dimensions, remains uncertain in the absence of detailed stereological analyses.

In summary, the reduction in nephrin expression in experimental diabetes as demonstrated in the present study suggests that modulation of this podocyte slit-membrane protein may be a determinant of glomerular hyperpermeability in diabetic nephropathy and possibly other renal diseases characterized by heavy proteinuria. Attenuation of these changes with ACE inhibition suggest that this mechanism may contribute to the anti-proteinuric effects of this but not all classes of drug which reduce proteinuria in diabetic nephropathy.

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Fig. 2. In situ hybridization photomicrographs of nephrin mRNA in (A) glomeruli of control rats, and in diabetic rats receiving (B) no treatment, (C) perindopril, or (D) aminoguanidine. Nephrin mRNA was noted exclusively in the glomerulus and predominantly in peripheral pattern of distribution consistent with its expression by podocytes (inset) (×1020).
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References


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