Original Article

P-Cadherin is decreased in diabetic glomeruli and in glucose-stimulated podocytes in vivo and in vitro studies

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Abstract

Background. Proteinuria is a cardinal feature of glomerular disease, including diabetic nephropathy, and the glomerular filtration barrier acts as a filter, restricting protein excretion in urine. We tested whether the expression of P-cadherin, a molecule known to be located at the slit diaphragm, was altered by diabetes *in vivo* and by high glucose *in vitro*.

Methods. *In vivo*, 24 Sprague–Dawley rats were injected with diluent [control (C), $n = 8$] or streptozotocin intraperitoneally and the latter were left untreated (DM, $n = 8$) or treated with insulin (DM + I, $n = 8$) for 6 weeks. *In vitro*, immortalized mouse podocytes were cultured in media with 5.6 mM glucose (LG), LG + 19.4 mM mannitol (LG + M) or 25 mM glucose (HG) with or without protein kinase C (PKC) inhibitor (10^{-7} M calphostin C or 10^{-6} M GF 109203X). Reverse transcription–polymerase chain reaction, western blotting for P-cadherin mRNA and protein expression, respectively, were performed with sieved glomeruli and cell lysates, and immunofluorescence staining was undertaken with renal tissue.

Results. Twenty-four hour urinary albumin excretion was significantly higher in DM compared with C and DM + I rats ($P < 0.05$). Glomerular P-cadherin mRNA expression was significantly lower in DM $(1.36 \pm 0.20 \times 10^{-2}$ attm/ng RNA) than in C rats $(2.61 \pm 0.33 \times 10^{-2}$ attm/ng RNA) $(P < 0.05)$. P-Cadherin protein expression, assessed by western blot and immunofluorescence staining, was also decreased in DM compared with C and DM + I glomeruli. HG significantly reduced P-cadherin mRNA and protein expression in cultured podocytes by 42% and 62%, respectively $(P < 0.05)$, and these decrements were ameliorated by PKC inhibitor.

Conclusions. Diabetes *in vivo* and exposure of podocytes to HG *in vitro* reduced P-cadherin mRNA and protein expression, and PKC was involved in the regulation of HG-induced down-regulation of P-cadherin. These findings suggest that the decrease in P-cadherin expression is connected with the early changes of diabetic nephropathy and, thus, may contribute to the development of proteinuria.

Keywords: P-cadherin; diabetic nephropathy; high glucose; podocyte; proteinuria

Introduction

Diabetic nephropathy, the leading cause of end-stage renal disease in the USA [1], is clinically characterized by proteinuria. The underlying pathological change responsible for proteinuria in various glomerular diseases, including diabetic nephropathy, is the loss of size-selective and/or charge-selective properties of the glomerular filtration barrier. The glomerular filtration barrier is comprised of three layers: a fenestrated endothelial layer, the glomerular basement membrane (GBM) and podocyte foot processes connected by a slit diaphragm. Traditionally, the GBM has been considered a coarse filter restricting large molecules, while the slit diaphragm was thought to function as a fine filter contributing ultimate size-selectivity, permitting permeability only to molecules smaller than albumin [2].

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The slit diaphragm, which bridges adjacent foot processes derived from different podocytes, is a continuous filamentous structure containing pores of 4×14 nm. In the past, the slit diaphragm was considered a modified tight junction due to the presence of the tight junction protein, zonula occludens-1, at the cytoplasmic side of the slit diaphragm [3]. However, recent studies using immunofluorescence and immuno-electron microscopy revealed that the slit diaphragm is a modified adherens junction rather than a modified tight junction and that P-cadherin was localized at the slit diaphragm [4]. In addition to P-cadherin, nephrin, NEPH1 and FAT are located in the slit diaphragm region [5].

Cadherins are a superfamily of glycoproteins that mediate Ca^{2+}-dependent, homotypic cell–cell adhesion in all solid tissues of the organism [6]. They are known to play important roles in maintaining the structural integrity of epithelial tissues and in tissue morphogenesis and to serve as signalling molecules to regulate cell behaviour, especially in some kinds of tumours. The classical cadherins are the best characterized members of the family and are designated by the originally described tissue specificity, including E- (epithelial), N- (neural) and P- (placental) cadherins [6]. E-Cadherin is expressed in most epithelial cells, whereas P-cadherin is restricted to basal cell layers, including basal cells of skin and prostate and myoepithelial cells of the mammary gland. Cadherins E and P have also been demonstrated in glomeruli [4,7], but their precise functions in the normal state and their changes in glomerulopathy have been explored less thoroughly. A recent study revealed that E-cadherin was expressed more frequently in glomerular epithelial cells and its expression in podocytes was slightly increased in patients with proliferative glomerulonephritis [7]. P-Cadherin is known to serve as a basic scaffold for the slit diaphragm, whereas the permselectivity is provided by the slit diaphragm complex composed of P-cadherin and other proteins, such as nephrin [4].

To investigate the pathogenesis of proteinuria in diabetic nephropathy, we tested whether the expression of P-cadherin was altered by diabetes *in vivo* and by high glucose in cultured podocytes *in vitro*. In addition, we determined whether protein kinase C (PKC), which is known to be activated under diabetic conditions in numerous cells, was involved in the changes in P-cadherin expression. In the current study, P-cadherin mRNA and protein levels were measured in glomeruli from streptozotocin-induced diabetic rats and in conditionally immortalized mouse podocytes cultured under high-glucose conditions.

Subjects and methods

Animals

All animal studies were conducted under a protocol approved by the committee for the care and use of laboratory animals of Yonsei University College of Medicine. Twenty-four male Sprague–Dawley rats, weighing 230–270 g, were studied. Eight were injected with diluent [control (C)] and 16 were injected with 65 mg/kg streptozotocin intraperitoneally. Blood glucose levels were measured on the third day after streptozotocin injection to confirm the development of diabetes. Diabetic rats were then randomly assigned to two groups. One group ($n = 8$) was treated with 2 U/day of Humulin (Ultralente; Eli Lilly Co., Indianapolis, IN, USA) (DM + I) and the remaining eight diabetic rats were left untreated (DM) to examine the effect of diabetes *per se*. In a previous study [8], diabetic rats without insulin were able to be maintained in good condition up to 4 months by keeping each rat in a separate cage and by changing cages every day. All rats were housed in a temperature-controlled room with free access to water and standard laboratory chow and were sacrificed after 6 weeks. When we measured the urinary albumin excretion in diabetic rats, a statistically significant increase in urinary albumin excretion was observed for the first time at 6 weeks after streptozotocin injection. Since the purpose of this study was to examine the changes of P-cadherin expression in a point of albuminuria, we used 6-week-old diabetic rats.

Body weights were checked weekly and kidney weights measured at the time of sacrifice. Serum glucose and 24 h urinary albumin also were measured at the time of sacrifice. Blood glucose was measured by glucometer and 24 h urinary albumin excretion was determined by enzyme-linked immunosorbent assay (Nephrat II; Exocell, Inc., Philadelphia, PA, USA).

Podocyte culture

Conditionally immortalized mouse podocytes were kindly provided by Dr Peter Mundel (Albert Einstein College of Medicine, Bronx, NY, USA) and were cultured as described previously [9]. Briefly, frozen podocytes were first grown under permissive conditions at 33°C in RPMI 1640 media containing 10% fetal bovine serum, 50 U/ml γ-interferon and 100 U/ml of penicillin/streptomycin in collagen-coated flasks and the γ-interferon was tapered down to 10 U/ml in successive passages. Cells were then trypsinized and subcultured without γ-interferon (non-permissive conditions) and allowed to differentiate at 37°C with media changed on alternate days. Differentiation of podocytes at 37°C was confirmed by the identification of synaptopodin, a podocyte differentiation marker, using reverse transcription–polymerase chain reaction (RT–PCR) and western blotting (data not shown).

After confirming differentiation of podocytes, medium was changed to RPMI medium containing normal glucose (5.6 mM; LG), LG + 19.4 mM mannitol (LG + M; to achieve isoosmolality) or high glucose (25 mM; HG) with or without 6 h pre-treatment of PKC inhibitor (10^{-7} M calphostin C or 10^{-6} M GF 109203X; Calbiochem, San Diego, CA, USA). After 3 days, cells were harvested for either RNA or protein.

Total RNA extraction

Glomeruli were isolated by sieving. The purity of the glomerular preparation was >98%, as determined by light microscopy. Total RNA was extracted as described previously [8]. Briefly, addition of 100 µl RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA) to the glomeruli was followed by glomerular lysis by freezing and thawing three

times. Another 700 µl RNA STAT-60 reagent was added, the mixture vortexed and stored for 5 min at room temperature; then, 400 µl chloroform was added and the mixture shaken vigorously for 30 s. After 3 min, the mixture was centrifuged at 12 000 g for 15 min at 4°C and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by 400 µl isopropanol and pelleted with centrifugation at 12 000 g for 30 min at 4°C. The RNA precipitate was washed with 70% ice-cold ethanol, dried using Speed Vac and dissolved in DEPC-treated distilled water. RNA from podocytes was extracted similarly. Glomerular and podocyte RNA yields and quality were assessed based on spectrophotometric measures at 260 and 280 nm.

Reverse transcription

First strand cDNA was made by utilizing a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Two µg of total RNA extracted from sieved glomeruli was reverse transcribed using 10 µM random hexanucleotide primer, 1 mM dNTP, 8 mM MgCl$_2$, 30 mM KCl, 50 mM Tris–HCl (pH 8.5), 0.2 mM dithiothreitol, 25 U RNase inhibitor and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 min and 42°C for 1 h, followed by inactivation of the enzyme at 99°C for 5 min. Podocyte RNA from each plate was similarly reverse transcribed.

Polymerase chain reaction

The primers used for rat and mouse GAPDH were as follows: rat sense, 5′-GACAAGATGGTGAAGGTCGG-3′, antisense 5′-CATGGACTGTGGTCATGAGC-3′; mouse sense, 5′-CCTGCACCACCAACTGCTTAGC-3′, antisense 5′-CCAGTGAGCTTCCCGTTCAGC-3′. Since the rat P-cadherin mRNA sequence has not been reported in GenBank, a partial sequence of rat P-cadherin mRNA was sequenced from the PCR product using cDNA of rat glomeruli and primers designed on the area where human and mouse P-cadherin showed homology (sense 5′-CTTCTGTGATGCAGGTGACAG-3′, antisense 5′-GCCTCAAAATCCAAACCCTTC-3′). Then, new rat and mouse P-cadherin primers were designed as follows: rat sense 5′-CTTACAATGGGGTGGTGG-3′, antisense 5′-GCCACGGTGAAATGATCC-3′; mouse sense 5′-CTTACAATGGGGTGGTGG-3′, antisense 5′-GTGATGGTGAAATGGTCC-3′. The cDNAs from 10 ng RNA of glomeruli and podocytes per reaction tube were used for GAPDH and 50 ng RNA of glomeruli and 20 ng RNA of podocytes for P-cadherin.

For GAPDH and P-cadherin mRNA expression, we used quantitative competitive PCR. The competitor cDNA was used as internal standard and was designed to contain the same base-pair sequence as the target cDNA that would allow efficient priming, but with a portion deleted so that the competitor PCR-generated fragment could easily be distinguished electrophoretically by size. The RT–PCR products were separated by electrophoresis, the band densities analysed by TINA image software (Raytest, Straubenhardt, Germany), the values log transformed and a log-linear regression analysis was performed against the competitor concentration for each PCR tube. The quantity of cDNA in the test sample was defined as the amount at

which the competitor and wild-type optical density bands were equal. PCR was performed using cDNA, 1.25 U *Taq*Gold polymerase, 20 µM dNTP and 25 pmol sense and antisense primers in a volume of 50 µl containing 1× PCR buffer. The PCR conditions were as follows: GAPDH, 35 cycles, denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min; and P-cadherin, 45 cycles for glomeruli and 42 cycles for podocytes, denaturation at 95°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 1 min. Initial heating at 95°C for 9 min and final extension at 72°C for 7 min was performed for all PCRs.

Western blot analysis

Sieved glomeruli and podocytes harvested from plates were lysed in sodium dodecyl sulphate (SDS) sample buffer [2% SDS, 10 mM Tris–HCl, pH 6.8, 10% (vol/vol) glycerol]. Lysate was centrifuged at 10 000 g for 10 min at 4°C and the supernatant was stored at −70°C until all rats were sacrificed. Protein concentrations were determined with a Bio-Rad kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Aliquots of 50 µg protein were treated with Laemmli sample buffer, then heated at 100°C for 5 min and electrophoresed (50 µg/lane) in an 8% acrylamide denaturing SDS–polyacrylamide gel. Proteins were transferred to Hybond–ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA) using a Hoeffer semi-dry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), the membrane incubated in blocking buffer A (1× PBS, 0.1% Tween-20 and 8% non-fat milk) for 1 h at room temperature and then incubated overnight at 4°C with a 1:500 dilution of monoclonal anti-P-cadherin antibody (Zymed Laboratories, Inc., San Francisco, CA, USA). The membrane was washed once for 15 min and twice for 5 min in 1× PBS with 0.1% Tween-20 and incubated in buffer A with horseradish peroxidase-linked sheep anti-mouse IgG (Amersham Life Science, Inc., Arlington Heights, IL, USA) at 1:1000 dilution. The washes were repeated and the membrane developed with chemiluminescent agent (ECL; Amersham Life Science, Inc., Arlington Heights, IL, USA).

Pathology

Slices of kidney for immunofluorescence staining were fixed in 10% neutral buffered formalin, processed in the standard manner and 5-µm sections of paraffin embedded tissues were utilized. Slides were deparaffinized, hydrated in ethyl alcohol and washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 min using a Black and Decker vegetable steamer. For P-cadherin staining, monoclonal anti-P-cadherin antibody (Zymed Laboratories, Inc., San Francisco, CA, USA) was diluted 1:200 with 2% casein in bovine serum albumin (BSA) and was applied for overnight incubation at room temperature. After washing, a secondary rhodamine red-X-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added for 60 min. A semi-quantitative score for measuring P-cadherin immunofluorescence intensity within glomeruli was determined by examining 30 glomeruli in each section and by digital image analysis (MetaMorph version 4.6r5; Universal Imaging Corp., Downingtown, PA, USA). To confirm that the change of P-cadherin expression was not due to the change of podocyte numbers, immunohistochemical

staining for Wilms' tumour-1 protein (WT-1) was also done. A rabbit polyclonal antibody to WT-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was diluted in 1:100 with 2% casein in BSA and was applied for overnight incubation at room temperature. After washing, a secondary goat anti-rabbit antibody was added for 20 min, the slides were washed and incubated with a tertiary rabbit–PAP complex for 20 min. DAB was added for 2 min and the slides were counterstained with haematoxylin. All cells stained positive for WT-1 in 20 glomeruli cut at the vascular pole were considered as podocytes and were counted to confirm the change of podocyte numbers.

Statistical analysis

All values are expressed as means ± SEM. Statistical analysis was performed using the statistical package SPSS for Windows® v. 7.51 (SPSS, Inc., Chicago, IL, USA). Results were analysed using the Kruskal–Wallis non-parametric test for multiple comparisons. If there was a significant difference by the Kruskal–Wallis test, it was confirmed further by the Mann–Whitney U-test. Statistical significance was determined when P-values were <0.05.

Results

Animal studies

Animal data. Body weight increased in all the three groups, but increased more in C (423 ± 5 g) than in DM (274 ± 4 g) and DM + I rats (356 ± 7 g) ($P < 0.01$). Kidney weight was measured at the time of sacrifice. Kidney weight and the ratio of kidney weight to body weight in DM rats (3.09 ± 0.10 g and $1.13 \pm 0.05\%$, respectively) were significantly higher than those in C rats (2.78 ± 0.06 g and $0.66 \pm 0.02\%$, respectively) ($P < 0.05$).

The mean blood glucose levels of C, DM and DM + I rats were 97.8 ± 2.2, 466.5 ± 5.2 and 254.2 ± 3.8 mg/dl, respectively ($P < 0.01$). Compared with the C group (0.32 ± 0.02 mg/day), 24 h urinary albumin excretion at 6 weeks was significantly higher in DM rats (1.18 ± 0.11 mg/day, $P < 0.05$) (Table 1).

Rat P-cadherin sequence. The PCR product amplified using cDNA of rat glomeruli and primers designed on the area where human and mouse P-cadherin showed homology was extracted from the gel and sequenced with ALFexpress DNA sequencer (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The partial rat P-cadherin sequence, excluding the primer regions, was compared with human and mouse P-cadherin sequences. Rat P-cadherin showed 90.7% homology with mouse P-cadherin and 84.2% homology with human P-cadherin (Figure 1).

Glomerular P-cadherin mRNA and protein expression. For the animal studies, we examined whether glomerular P-cadherin mRNA and protein expressions were altered in DM rats and whether the changes in P-cadherin expression were associated with the development of albuminuria. Glomerular P-cadherin mRNA was significantly lower in DM ($1.36 \pm 0.20 \times 10^{-2}$ attm/ng RNA) than C rats ($2.61 \pm 0.33 \times 10^{-2}$ attm/ng RNA) ($P < 0.05$) at 6 weeks after induction of DM, while the amount in the DM + I was intermediate ($2.27 \pm 0.32 \times 10^{-2}$ attm/ng RNA) (Figures 2A and 2B). In contrast, there was no significant difference in the amount of glomerular GAPDH mRNA among the three groups (data not shown). Even when corrected for GAPDH mRNA, glomerular P-cadherin mRNA remained significantly lower in DM compared with C (P-cadherin/GAPDH: $0.51 \pm 0.06 \times 10^{-1}$ vs $1.16 \pm 0.21 \times 10^{-1}$; $P < 0.05$).

Figure 3 shows a representative western blot of equal amounts of protein from the lysates of sieved C, DM and DM + I glomeruli at 6 weeks. Glomerular P-cadherin protein expression was also decreased in DM compared with C and DM + I rats as P-cadherin mRNA expression. Densitometric quantitation revealed that there was a 67% decrease in P-cadherin protein expression in DM relative to C rats ($P < 0.01$), with no difference in β-actin protein expression.

Pathology. Immunofluorescence staining for glomerular P-cadherin confirmed the mRNA and western blot findings. There was a significant decrease in glomerular P-cadherin expression, which exhibited linear/punctate distribution along the glomerular capillary loops, in DM rats. The decrease in P-cadherin expression in DM glomeruli was ameliorated by insulin treatment. The semi-quantitative score for immunofluorescence intensity for P-cadherin was significantly lower in DM compared with C ($P < 0.01$) and DM + I glomeruli ($P < 0.05$) (Figures 4A and 4B). In contrast, there was no statistical difference in mean podocyte counts per glomerulus assessed by WT-1 staining among the three

Table 1. Body weight, kidney weight, kidney weight/body weight, blood glucose and 24 h urinary albumin excretion of the three groups

	Control ($n = 8$)	DM ($n = 8$)	DM + I ($n = 8$)
Body weight after 6 weeks (g)	423 ± 5	274 ± 4[a]	356 ± 7[a,b]
Kidney weight (g)	2.78 ± 0.06	3.09 ± 0.10[c]	2.81 ± 0.08[d]
Kidney wt/body wt (%)	0.66 ± 0.02	1.13 ± 0.05[c]	0.79 ± 0.04[d]
Blood glucose (mg/dl)	97.8 ± 2.2	466.5 ± 5.2[a]	254.2 ± 3.8[a,b]
24 h urinary albumin excretion (mg/day)	0.32 ± 0.02	1.18 ± 0.11[c]	0.62 ± 0.08[d]

[a] $P < 0.01$ vs control.
[b] $P < 0.01$ vs DM.
[c] $P < 0.05$ vs control.
[d] $P < 0.05$ vs DM.

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Rat:           CCACAGATGAGGACGACGCCATCAATACTTACAATGGGGTGGTGGCT
Mouse:   737-  CCACAGATGAGGACGATGCTGTCAACACTTACAATGGGGTGGTGGCT
Human:   769-  CCACAGATGAGGATGATGCCATCTACACCTACAATGGGGTGGTTGCT

               TACTCCATCCATAGCCAAGAGCCGAAGGAGCCACACGACCTCATGTTCACCATCCATAAA
784-           TACTCCATCCATAGCCAAGAGCCGAAGGAGCCACACGACCTCATGTTCACCATCCATAAA
816-           TACTCCATCCATAGCCAAGAACCAAAGGAGCCACACGACCTCATGTTCACAATTCACCGG

               AGCACTGGGACTATTAGCGTCATCTCCAGTGGCCTGGATAGAGAGAAAGTCCCTGAGTAC
844-           AGCACGGGAACCATTAGCGTCATATCCAGTGGCCTGGACCGAGAGAAAGTCCCTGAGTAC
876-           AGCACAGGCCACCATCAGCGTCATCTCCAGTGGCCTGGACCGGGAAAAAGTCCCTGAGTAC

               ACACTGACCATCCAGGCCACAGACATGGACGGAGACGGCTCTACCACAACAGCCGAGGCC
904-           AGACTGACCGTCCAGGCCACAGACATGGATGGAGAGGGCTCTACCACGACGGCAGAGGCC
936-           ACACTGACCATCCAGGCCACAGACATGGATGGGGACGGCTCCACCACCACGGCAGTGGCA

               GTAGTGCAGATCCTTGATACCAATGATAACGCTCCAGAGTTCCAGCCGCAAAAGTATGAG
964-           GTTGTGCAAATCCTTGATGCCAACGATAACGCTCCGGAGTTTGAGCCGCAGAAGTATGAG
996-           GTAGTGGAGATCCTTGATGCCAATGACAATGCTCCCATGTTTGACCCCCAGAAGTACGAG

               GCTTGGGTGCCTGAGAATGCAGTGGGCCACGAGGTACAGAGGCTGACAGTGACCGATCTG
1024-          GCTTGGGTGCCTGAGAACGAAGTGGGCCATGAGGTACAGAGGCTGACAGTGACTGATCTC
1056-          GCCCATGTGCCTGAGAATGCAGTGGGCCATGAGGTGCAGAGGCTGACGGTCACTGATCTG

               GATTCCCCCAACTCACCGGCATGGCGTGCCACCTACCACATTGTGGGAGGTGACGATGGG
1084-          GATGTCCCCAACTGGCCATGCGTGGCGTGCCACCTACCACATCGTGGGAGGTGATGATGGG
1116-          GACGCCCCCAACTCACCAGCGTGGCGTGCCACCTACCTTATCATGGGCGGTGACGACGGG

               GATCATTTCACCGTGGCCACTCACCCAGAGACCAACCAAGGCATCCTGACAACCAA
1144-          GACCATTTCACCATCACCACTCACCCAGAGACCAACCAAGGCGTCCTGACAACCAA
1176-          GACCATTTTACCATCACCACCCACCCTGAGAGCAACCAGGGCATCCTGACAACCAA
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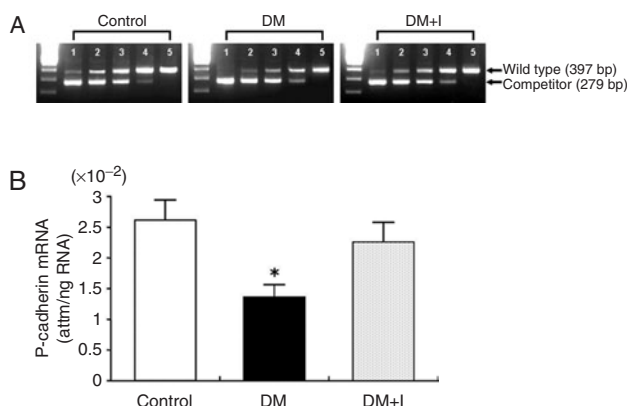
Fig. 1. The partial nucleotide sequence of rat P-cadherin. Rat P-cadherin showed 90.7% homology with mouse P-cadherin and 84.2% homology with human P-cadherin. The grey box indicates the unconserved nucleotide shared by rat and mouse or by rat and human. The nucleotides used as primers for RT–PCR are underlined.
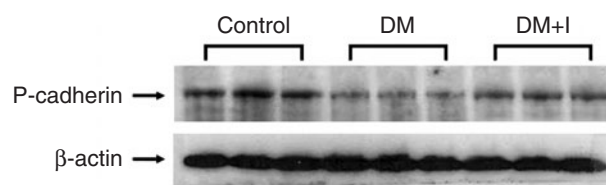

Fig. 2. Glomerular P-cadherin and GAPDH mRNA expressions in C and DM rats. (**A**) A representative P-cadherin competitive RT–PCR electrophoresis of C, DM and DM+I glomeruli at 6 weeks after DM induction ($n=8$). Each lane represents a competitive RT–PCR reaction (45 cycles) with a fixed amount of P-cadherin wild-type cDNA (397 bp) from the equivalent of 50 ng RNA and a variable amount of P-cadherin competitor cDNA (279 bp) as follows: (lane 1) 0.5 attm/µl, (lane 2) 0.1 attm/µl, (lane 3) 0.05 attm/µl, (lane 4) 0.01 attm/µl and (lane 5) 0.005 attm/µl. (**B**) A significant decrement in glomerular P-cadherin mRNA expression was observed in DM *vs* C rats. *$P<0.05$ *vs* C and DM+I.

Fig. 3. A representative P-cadherin western blot of C, DM and DM+I glomeruli at 6 weeks after DM induction. Densitometric quantitation revealed that there was a 67% decrease in P-cadherin protein expression in DM relative to C rats ($P<0.01$), whereas there was no difference in β-actin protein expression.

groups (C: 15.8 ± 1.8; DM: 15.3 ± 1.9; DM+I: 16.1 ± 2.0).

Podocyte culture studies

*P-Cadherin mRNA and protein expression.* The P-cadherin mRNA of podocytes exposed to HG was significantly lower than that of podocytes in LG media (HG: $0.89 \pm 0.10 \times 10^{-2}$ attm/ng RNA; LG: $1.58 \pm 0.28 \times 10^{-2}$ attm/ng RNA; $P<0.05$) and this HG-induced decrement in P-cadherin mRNA expression was ameliorated by the PKC inhibitors calphostin C ($1.39 \pm 0.22 \times 10^{-2}$ attm/ng RNA) or GF 109203X ($1.32 \pm 0.32 \times 10^{-2}$ attm/ng RNA) (Figure 5). On the other hand, there was no significant difference in the amount of GAPDH mRNA (data not shown). After correction for GAPDH mRNA, the differences in P-cadherin mRNA expression remained significant (P-cadherin/GAPDH: $2.69 \pm 0.26 \times 10^{-2}$ *vs* $1.52 \pm 0.25 \times 10^{-2}$; $P<0.01$).

The P-cadherin protein expression also was significantly lower in HG than in LG podocytes. There was a 62% decrease in P-cadherin protein expression in HG compared with LG cells assessed by densitometry ($P<0.05$) and PKC inhibitor nearly normalized
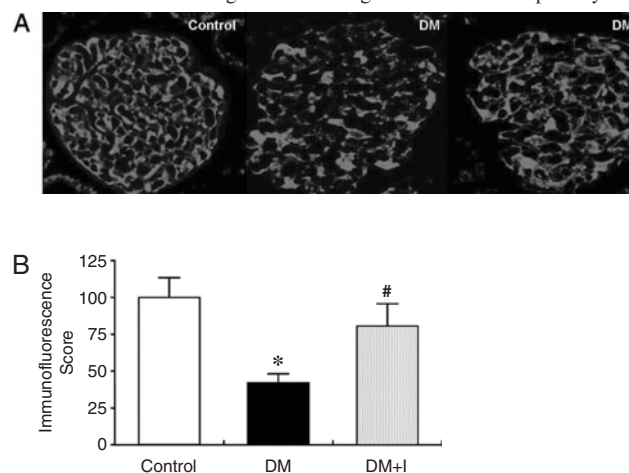
Fig. 4. Immunofluorescence staining for P-cadherin protein in C, DM and DM+I glomeruli at 6 weeks after DM induction. (**A**) There was a significant decrease in glomerular P-cadherin expression, which exhibited linear/punctate distribution along the glomerular capillary loops, in DM rats. The decrease in P-cadherin expression in DM glomeruli was ameliorated by insulin treatment. (Magnification: ×400.) (**B**) Semi-quantitative immunofluorescence score for glomerular P-cadherin was significantly lower in DM compared with C rats. *$P < 0.01$ vs C; #$P < 0.05$ vs DM.
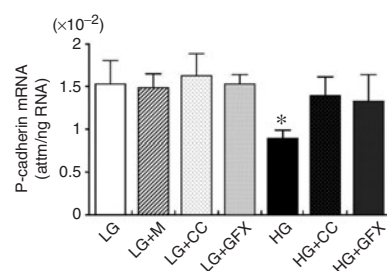

Fig. 5. P-Cadherin and GAPDH mRNA expressions in podocytes exposed to LG, LG+M or HG medium with or without PKC inhibitor, 10^{-7} M calphostin C (CC) or 10^{-6} M GF 109203X (GFX) ($n = 5$). A significant decrement in P-cadherin mRNA expression was observed in HG cells compared with LG cells and this HG-induced decrement in P-cadherin mRNA expression was ameliorated by PKC inhibitor. Mannitol (M) had no effect on the P-cadherin mRNA expression of podocytes. *$P < 0.05$ vs LG and HG+PKC inhibitor.

this HG-induced reduction in P-cadherin protein expression (Figure 6).

Mannitol (19.4 mM) had no effect on the P-cadherin mRNA and protein expression of podocytes.

Discussion

Diabetic nephropathy is characterized pathologically by glomerular and tubular hypertrophy and increased extracellular matrix accumulation and clinically by proteinuria. The underlying pathological change responsible for proteinuria in various glomerular diseases is the loss of size-selective and/or charge-selective properties of the glomerular filtration barrier [2], which is comprised of glomerular endothelium,
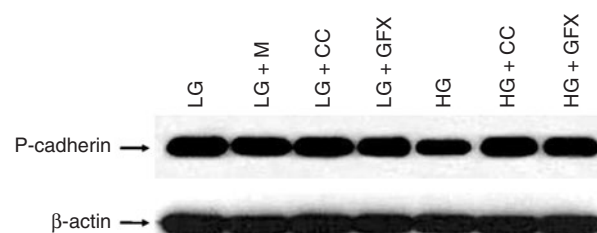
Fig. 6. Western blot of P-cadherin in podocytes exposed to LG and HG for 5 days (representative of five blots). P-Cadherin protein expression was significantly lower in podocytes exposed to HG than in those exposed to LG. Densitometric quantitation revealed that there was a 62% decrease in P-cadherin protein expression in podocytes cultured under HG conditions vs those cultured under LG conditions ($P < 0.05$) and this HG-induced decrement in P-cadherin protein expression was ameliorated by PKC inhibitor, 10^{-7} M calphostin C (CC) or 10^{-6} M GF 109203X (GFX). Mannitol (M) had no effect on the P-cadherin protein expression of podocytes.

basement membrane and podocytes connected by a slit diaphragm. In this study, we demonstrate for the first time that P-cadherin mRNA and protein expression are decreased in experimental diabetic glomeruli and in high-glucose-stimulated podocytes, suggesting a potential role for P-cadherin loss in the development of proteinuria in early diabetic nephropathy.

It has been suggested that changes in these glomerular filtration barrier-associated molecules play critical roles in the contribution to the pathogenesis of proteinuria in glomerular disease. Based on this view, there have been several reports on the changes of these filtration barrier-associated molecules in various kinds of kidney diseases, but most of the previous studies were focused on changes in nephrin [10–12], a product of the *NPHS1* gene, which is mutated in patients with congenital nephrotic syndrome of the Finnish type [13]. Since proteinuria is a cardinal feature of diabetic nephropathy, the contribution of changes in nephrin expression to proteinuria in diabetic nephropathy has also been studied, but the results were not consistent. Bonnet *et al.* [12] demonstrated a reduction in both mRNA and protein expression of nephrin in diabetic spontaneously hypertensive rats, whereas Aaltonen *et al.* [11] observed an increase in nephrin mRNA levels in streptozotocin-induced diabetic rats and in non-obese diabetic mice even before significant albuminuria developed. The reasons for the divergence of changes in nephrin expression in diabetic nephropathy may be due to a variety of underlying causes, including but not limited to species differences, differences in diabetes duration or accompanied hypertension.

P-Cadherin is one of the classical cadherins, a superfamily of glycoproteins involved in cell–cell adhesion, and is known to exist in the basal cells of skin and prostate, in the myoepithelial cells of the mammary gland and in the glomerular slit diaphragm [4,6]. Based on the location of P-cadherin at the slit diaphragm, it has been proposed that P-cadherin serves as a basic scaffold for the slit diaphragm, whereas the permselectivity is provided by the slit diaphragm

complex composed of P-cadherin and other proteins, such as nephrin [4]. In contrast to nephrin, the role of P-cadherin in the pathogenesis of proteinuria has been studied less. Bains *et al.* [10] failed to demonstrate any changes of cadherin, including P-cadherin protein expression, using Pan-cadherin antibody in patients with minimal change nephrotic syndrome and membranous glomerulonephritis assessed by indirect immunofluorescence microscopy. Because cadherin was not detected in either the normal or the diseased glomeruli by indirect immunofluorescence, it was difficult to identify minor changes of cadherin expression in proteinuric patients. In congenital Finnish type nephrotic kidney, P-cadherin was expressed normally in spite of absent slit diaphragms [14]. In addition, even though proteinuria was not investigated in P-cadherin-deficient mice, it seemed that life-threatening proteinuria did not develop in view of the survival of these mice [15]. In contrast, a more recent study demonstrated that intravenous injection of anti-P-cadherin antibody resulted in a 49% increase in 24 h urinary protein excretion independent of nephrin or NEPH1 [16], suggesting that P-cadherin also serves as a glomerular filtration barrier to protein. As described previously, the mRNA expression of nephrin was increased rather than decreased in early diabetic nephropathy [11] and, in the present study, we demonstrated a decrease in P-cadherin mRNA and protein expression in 6-week-old diabetic glomeruli. Therefore, the potential role of P-cadherin in the development of proteinuria in diabetes may not be an irrelevant finding and it seems likely that the molecular changes of the slit diaphragm complex, and not a single slit diaphragm-associated molecule, might contribute to the development of proteinuria in diabetic nephropathy.

The numbers of glomerular podocytes are known to decrease in type 1 diabetic patients of all ages, with reduced podocyte numbers even in diabetes of short duration [17]. Analysis of kidney biopsies from Pima Indians with type 2 diabetes also demonstrated that subjects with clinical nephropathy exhibited broadening of podocyte foot processes associated with a reduction in the number of podocytes per glomerulus [18]. Focal detachment of podocytes from the GBM may be associated with a decrease in $\alpha3\beta1$ integrin at the podocyte basal plasma membrane, which occurs as early as 1 month after the onset of hyperglycaemia [19]. In the present study, P-cadherin mRNA expression was significantly decreased in HG-stimulated podocytes, whereas there was no difference in GAPDH mRNA expression. In addition, immunofluorescence microscopy revealed a reduction of P-cadherin staining, both in intensity and area, in diabetic glomeruli with no significant difference in podocyte numbers assessed by WT-1 staining. These findings suggest that the reduction of P-cadherin mRNA and protein expression in early diabetic conditions is not attributed to the decrease in podocyte numbers.

Besides their basic role in cell–cell adhesion, cadherins appear to be involved in cellular signalling and differentiation [6]. Since little is known about

the role of P-cadherin in signal transduction in the glomerular podocytes, it is uncertain whether the decrease in P-cadherin expression under diabetic conditions is a direct cause of proteinuria in diabetic nephropathy or a coincident change associated with the signal transduction pathway. Numerous studies have revealed that PKC is activated in diabetic conditions, *in vivo* and *in vitro* [20]. In addition, the PKC pathway is a key signal pathway associated with the activation of transforming growth factor-$\beta1$ and fibronectin synthesis, which are important in the pathogenesis of diabetic nephropathy [20]. In this study, P-cadherin expression was decreased in cells exposed to HG and this decrement was ameliorated by the PKC inhibitor, suggesting that P-cadherin expression is closely linked to activation of the PKC pathway.

To conclude, in diabetes *in vivo* and in podocytes cultured in HG, P-cadherin mRNA and protein expressions are decreased and PKC seems to be involved in HG-induced down-regulation of P-cadherin expression. In addition, the changes in glomerular P-cadherin expression are associated with the development of albuminuria in diabetic rats. Taken together, the data suggest that alterations in P-cadherin in podocytes are related to early changes of diabetic nephropathy and, thus, may contribute to the development of proteinuria.

Conflict of interest statement. None declared.

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