Molecular mechanisms of podocyte damage in diabetes mellitus

Doctoral theses

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1 INTRODUCTION

Glomerular visceral epithelial cells, namely podocites, play an important role in maintaining the function and structure of glomerular filtration barrier. Observations on human and experimental models identify podocyte damage as an early indicator diabetic nephropathy (DN), but the molecular mechanisms of podocyte damage are not completely understood, therapy is yet to be solved.

1.1 Selective phosphodiesterase 5 inhibition in diabetic nephropathy

In diabetes, the increasing intraglomerular pressure and the resulting glomerular hypertension can be ameliorated by affecting cyclic guanosine monophosphate (cGMP) nitrogen monoxide (NO) axis. As an effect of NO renal blood vessels dilate, including afferent arterioles as well. Intracellular cGMP mediates the effect of NO, which induces vascular smooth muscle relaxation. Among hemodynamic actions, NO-driven cGMP regulates glomerular filtration by modulating the slit membrane and cytoskeletal reorganization in podocytes. cGMP is degraded by phosphodiesterase-5 (PDE5) enzyme, and vascular cGMP pathway can be stimulated by PDE5 inhibitors. PDE5 inhibitors (sildenafil, tadalafil, vardenafil) increase cGMP levels, and are main indications for treating erectile dysfunction as they promote relaxation of penile blood vessels and so the corpus cavernosum can be easily saturated. The PDE5 enzyme is abundantly present in the kidneys. The activity of phosphodiesterases increases in nephropathy, cGMP levels reduce consequently, and thus indirectly the NO effect decreases. Using PDE inhibitors, cGMP levels can be normalized. The increased cGMP levels may enhance the impact of NO and therefore slow down the progression of glomerulosclerosis. A number of research is based on this idea nowadays, that examine the mechanism of different PDE inhibitors in various nephropathies. Data about the activity of phosphodiesterases are limited in DN, although their results may encourage the use of phosphodiesterase inhibitors in the treatment of diabetic nephropathy or other nephropathies.

The NO-cGMP system is damaged at several points in DN: insufficient eNOS function due to oxidative stress; deactivated NO by reactive oxygen species; reduced NO penetrating ability through the thickened endothelial basement membrane.

Furthermore, degradation of cGMP is accelerated as a result of oxidized sGC dysfunction and increased PDE catabolizing activity. The effect of NO dependent cGMP is reduced, ultimately.

In our study, we investigated whether the elevation of cGMP levels by pharmacological treatment with vardenafil, a highly selective PDE-5 inhibitor could preserve podocyte function and reduce podocyte damage in the rat model of streptozotocin (STZ)-induced type 1 diabetes mellitus. We found that vardenafil treatment raised serum cGMP levels and intracellular cGMP levels in podocytes, decreased proteinuria, attenuated podocyte damage and restored nephrin and podocin expression.

1.2 The role of SCAI diabetic nephropathy

In DN research, beside therapy, much attention is paid to learn new signaling pathways. SCAI – suppressor of cancer cell invasion – is a newly identified transcriptional cofactor, which seems to be promising in the regulation of malignant tumour diseases. There is close relationship between SCAI and Wnt/beta-catenin pathway in the pathogenesis of glioma, furthermore, the progression of glioma changes according to the expression of SCAI. Wnt signaling pathway is overactivated in cancerous processes as well as in type 1 and type 2 diabetes mellitus. If the SCAI and Wnt/beta-catenin pathway are linked, the encouraging effects of SCAI in tumour diseases raise the question, how would SCAI molecule effect the pathogenesis of DN.

The previously hypothetical protein, SCAI was first described in 2009 by Brandt et al., regulates invasive cell migration. SCAI has a highly conserved structure and showa a wide tissue distribution among vertebrates. In the absence of SCAI, the expression of ß1 integrin gene is increased extremely, that is a cell surface adhesion receptor. The integrins induce actin accumulation, directly activate actin polymerization and bind the actin cytoskeleton to the extracellular matrix. α-smooth muscle actin (α-SMA), one of the six actin isoforms, plays a significant role in fibrosis and is the most typical marker of fibrosis. Renal interstitial upregulation of α-SMA activates myofibroblasts, that correlates with the extent of interstitial fibrosis. Wnt/beta-catenin signaling is involved in epithelial-mesenchymal transition (EMT) process of mesangial cells in diabetes. TGF-ß is an important mediator in the development of high blood

sugar caused EMT, in that microRNAs (miRNA) also play a role. MiRNAs are short (about 20-24 nucleotides) non-coding RNA molecules, that are linked with partially complementary mRNAs and induce the breakdown of target molecules, thus silencing their expression. Beta-catenin-mediator protein-1 (CTNNBIP1) is the target molecule of miRNA-215. In diabetes, increased miRNA-215 expression inhibits CTNNBIP1, and activates Wnt/beta-catenin pathway, that results the progression of TGF-ß mediated EMT in mesangial cells and the increase of fibronectin and α-SMA expression. The expression of integrins, and the number of α-SMA positive myofibroblasts increase and play crucial roles in the pathogenesis of renal fibrosis, but the expression of SCAI, that is affecting assumingly upstream these processes, has not yet been studied in renal fibrosis so far.

We assumed that in conditions where α-SMA expression is found high (renal fibrosis), the expression of SCAI might be low. We examined mRNA and protein expression and tissue localization of SCAI in diabetic nephropathy model. Our experiments suggest that SCAI is involved in renal fibrosis by inhibiting α-SMA expression, the significant myofibroblast marker.

2 OBJECTIVES

To study the pathomechanism of diabetic nephropathy and molecular mechanism of podocyte damage our aims were the following:

- Is it possible to affect the progression of type I diabetic nephropathy in rats with pharmacotherapeutic support of NO-cGMP pathway by vardenafil?
- To what changes will vardenafil treatment lead on tissue and molecular level in the diabetic kidney?
- What is the cGMP expression of podocyes in diabetes without treatment or with vardenafil treatment?
- What is the SCAI expression in diabetic kidney?
- How does hyperglycaemia affect SCAI expression of podocytes in vitro?

3 METHODS

3.1 Animals, induction of diabetes mellitus

Type 1 diabetes mellitus was induced in male Sprague-Dawley rats (250-300g) with a single intraperitoneal dose of streptozotocin (STZ) dissolved in citrate buffer (0.1 mol/L). Control animals received buffer only. After 72h, animals with random blood glucose level >15mmol/l were considered as diabetic and were included into the study. Diabetic rats were randomized to diabetic control (STZ, n=6) and vardenafil treatment (STZ-Vard, n=8) groups. Rats injected only with citrate buffer served as non-diabetic controls (Control, n=7). Diabetic animals were treated for 8 weeks with the selective phosphodiesterase-5 inhibitor, vardenafil (STZ-Vard group, 10mg/kg/day dissolved in 0.01 mol/l citrate buffer) or with vehicle (Control and STZ groups) per os in drinking water. The daily water intake was registered and the dose of vardenafil was repeatedly adjusted. Body and kidney weights were measured at the time of harvest.

To characterize the role of SCAI in diabetic nephropathy, after induction of diabetes rats were separated into two groups: Diabetic (n=4) and Control (n=4). Nondiabetic controls were injected only with citrate buffer, instead of streptozotocin.

For further analysis of SCAI expression, diabetes was induced in male FVB/N mice (n=8) at the age of 8 weeks, with daily i.p. injections of STZ for 5 days. Control mice (n=7) received citrate buffer only. One week later, STZ-injected mice with a fasting blood glucose level <20 mmol/L were excluded from the study (n=3). Mice were sacrificed 8 weeks after the induction of diabetes, and kidneys were analyzed.

3.2 Blood pressure measurements, blood and urine chemistries

At the end of the treatment period, rats were anesthetized and were placed on controlled heating pads. Arterial blood pressure was recorded by 2F microtip pressure-volume catheter, and mean arterial pressure (MAP) was computed.

Serum glucose and urea levels as well as urine creatinine concentration were determined photometrically on a Reflotron analyzer. Urine protein concentration was measured using the BCA Protein Assay, and urinary protein/creatinine ratios were calculated.

Serum cyclic guanosine monophosphate (cGMP) levels were determined by enzyme immunoassay (EIA) using a commercial kit.

3.3 Renal histology

Periodic-acid Schiff (PAS) staining was performed on formalin fixed, paraffin embedded kidney samples. Kidney damage was evaluated by scoring systems of glomerular damage (grade 0-4) and tubular damage (grade 1-5). The glomerular score of each animal was derived as the arithmetic mean of 60 glomeruli (400x magnification). The tubular damage was assessed at 100x magnification of the light microscopy. Samples were evaluated in blinded manner.

3.4 Immunhistochemistry

Immunohistochemistry was performed on paraffin sections, using the avidin-biotin method. Samples were blocked with goat serum, and slides were incubated with each primary antibody (fibronectin, TGF-β_1, desmin, nephrin, nitrotyrosine, cGMP, SCAI). After washing, the appropriate biotinylated secondary antibodies and consecutively streptavidine-conjugated alkaline phosphatase was applied. Slides were developed using Fast Red substrate system. Immunohistochemical reactivity was evaluated in a blinded manner using a semiquantitative scoring system (grade 0-4).

Nephrin and cGMP double immunostaining was performed on frozen kidney sections. Acetone fixed sections were blocked using donkey serum, then incubated with primary antibodies (anti-cGMP, nephrin,). After washing with PBS, slides were incubated with fluorescent dye conjugated secondary antibodies. The sections were analyzed under fluorescent microscope.

3.5 Semiquantitative immunoblot

Kidney samples were homogenized in RIPA lysis buffer. Protein concentration was determined by the BCA Assay. Samples were mixed in 1:1 ratio with 2x Laemmli buffer and boiled. Equal amounts of protein were separated on 10% gel, transferred to nitrocellulose membranes and blocked with skim milk solution. Membranes were incubated overnight at 4°C with primary antibodies (PDE5a, SMA, GAPDH). Consecutively membranes were incubated with the appropriate peroxidase-conjugated secondary antibody. Blots were visualized by ECL detection kit.

3.6 Quantitative RT-PCR

Whole kidney samples were homogenized and total RNA was isolated. RNA integrity was checked. 2μg PCR reactions (rat: TGF-ß, eNOS, nNOS, nephrin, podocin, GAPDH; mouse: SCAI, GAPDH) were performed on a BioRad CFX thermal cycler, using random primers. Duplicate samples were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

3.7 Mouse podocyte cell line

In our study we used immortalized mouse podocytes, bearing thermosensitive SV40-T gene (Jeffrey Kopp, NIH). To propagate podocytes, cells were cultivated under permissive conditions. As the cells reached the state of 70% confluence, plates were separated into two groups (n=5 plates/group), and podocytes were maintained under nonpermissice conditions to induce differentiation. After 5 days of differentiation one group received medium containing 5 mM glucose (Normoglycaemic), the other group received medium containing 25 mM glucose (Hyperglycaemic). RNA was isolated using Trizol at 48h and 7 days.

3.8 Statistics

All the data are presented as mean ± SD. Data were tested for normal distribution. For data with normal distribution, groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Otherwise, data were analyzed using Kruskal-Wallis test. Level of significance was set to $p<0.05$.

4 RESULTS

4.1 Selective phosphodiesterase-5 inhibition ameliorated podocyte damage in diabetic rats by increasing cGMP levels

Effectiveness of vardenafil treatment was evaluated by serum cGMP levels at harvest. Diabetes tended to reduce serum cGMP levels in non-treated STZ rats as compared to non-diabetic controls, but vardenafil treatment significantly restored, and actually elevated serum cGMP levels above the range of non-diabetic controls.

Serum glucose levels and daily water intake rose markedly, while body weight decreased in diabetic rats, regardless of treatment. Kidneys of both vardenafil treated

and non-treated diabetic rats developed significant hypertrophy as shown by increased kidney weight/body weight ratio at time of harvest.

STZ rats tended to have slightly elevated serum urea levels, but the difference was statistically not significant. Vardenafil treatment had no effect on se urea levels. There was no difference in the mean arterial blood pressure (MAP) values among the groups at harvest.

Diabetes led to significantly elevated urine protein/creatinine ratio in STZ rats as compared to healthy controls. Vardenafil treated rats, however, had strikingly lower urinary protein/creatinine ratio as compared to STZ rats.

Renal histology of the experimental groups was analyzed using PAS staining on the kidney sections. When compared to controls, glomerular hypertrophy, mild mesangial expansion and adhesions to Bowman's capsule were observed in diabetic rats, which were ameliorated by vardenafil treatment. The tubulointerstitial lesions of STZ rats were characterized by tubular dilatation and atrophy, which was significantly ameliorated by vardenafil treatment. Mononuclear cell infiltration was not seen in control kidneys, and only to a minimal extent in diabetic kidneys regardless of treatment protocol.

Fibronectin expression, evaluated by immunohistochemistry, was augmented in STZ rats, but vardenafil treatment significantly reduced the expression of fibronectin in diabetic kidneys implying its antifibrotic effect.

TGF-β_1 mRNA expression in whole kidney homogenates was significantly higher in non-treated STZ rats as compared to non-diabetic controls. Vardenafil treatment normalized the mRNA expression to similar levels as seen in non-diabetic controls.

Both glomeruli and tubulointerstitium of STZ kidneys showed strong TGF-β_1 immunostaining. According to mRNA expression results, vardenafil treatment significantly ameliorated both glomerular and tubulointerstitial TGF-β_1 expression in diabetic rats, as compared to non-treated STZ.

Immunoblot analysis revealed striking renal PDE5 overexpression in both non-treated and treated diabetic rats as compared to controls.

Immunostaining for glomerular cGMP content revealed significant expression in control kidneys, mostly in the cytoplasm of podocytes, and to smaller extent in the

endothelial cells. There was a dramatic decrease in cGMP staining intensity in STZ rats as compared to non-diabetic controls. In contrast, vardenafil treatment almost restored glomerular cGMP content. Evaluation of tubular cGMP staining showed similar results, which shows that vardenafil could preserve intracellular cGMP levels in diabetic kidneys.

Double immunostaining depicted glomerular co-localization of cGMP and nephrin in controls and STZ-Vard kidneys. Compared to non-diabetic controls, glomerular cGMP staining intensity of STZ rats was dramatically reduced, and cGMP was practically absent in nephrin positive podocytes. In contrast, podocytes of vardenafil treated rats depicted apparently elevated cGMP content as compared to non-treated STZ rats. In 2-5% of cGMP positive areas there was no nephrin co-localization in the capillaries, which refers to the cGMP staining of endothelial cells.

To evaluate the extent of podocyte damage, desmin and nephrin immunostaining were performed. Desmin staining (as a marker of podocyte damage) was stronger in STZ group than in non-diabetic controls. Vardenafil significantly reduced desmin expression to an intermediate level between the STZ and the control levels. In contrast, nephrin staining was reduced in STZ rats as compared to non-diabetic controls, but restored after chronic vardenafil treatment. We wanted to further evaluate podocyte damage by measuring levels of nephrin and podocin mRNA expression. During progression, podocin and nephrin expressions become usually reduced in podocytes, and that is accompanied by increased proteinuria. In our study, both nephrin and podocin mRNA expression levels in diabetic rats were reduced by ~50% as compared to non-diabetic controls, but vardenafil treatment restored nephrin and podocin mRNA expression to normal levels.

Diabetic kidneys presented strong nitrotyrosine immunoreactivity (as a marker of nitrosoxidative stress) both in glomeruli and tubulointerstitium which supports the theory of increased local oxidative stress. PDE-5 inhibition with vardenafil had no significant influence on the amount of renal nitrotyrosine formation.

Expression of nNOS mRNA was significantly increased in diabetic groups regardless of treatment protocol. Although non-treated diabetic kidneys expressed slightly higher levels of eNOS mRNA than non-diabetic controls, the differences were

not statistically significant and vardenafil did not alter eNOS expression. The mRNA expression of iNOS was similar in all groups.

4.2 SCAI expression in diabetis nephropathy

Induction of diabetes led to significantly elevated urine protein/creatinine ratio and serum glucose and elevated serum urea levels in diabetic rats as compared to non-diabetic controls.

Diabetic animals developed glomerular and tubulointerstitial damage characterized by: mesangial expansion and tubular atrophy, hyaline deposits and mild mononuclear cell infiltration, corresponding to findings in an early phase of nephropathy.

To substantiate our findings, we next performed Western blots to test whether there is an inverse relationship of SCAI and SMA expression in control and diseased kidneys. For this control and diabetic rat medullas were used. There is an inverse relationship between SCAI and SMA expression: SCAI tends to be more elevated in control samples and its expression level declines in kidneys affected by diabetic nephropathy, whereas SMA expression was found more pronounced in diabetic samples.

Tubular cells in control kidneys showed more intense nuclear SCAI staining than tubular cells in diabetic kidneys.

Immunostaining of SCAI expression showed similar extent in podocytes of both control and diabetic rats. Altough there was a tendency of more intense SCAI staining in the diabetic glomeruli in comparison to the controls.

To evaluate mRNA expression of SCAI, kidneys of diabetic mouse were used. According to the findings of Western blot and immunohistochemistry in the rat experiment, SCAI mRNA expression was significantly – approximately 50% - lower in diabetic kidneys compared to healthy controls.

Since the SCAI immunostaining of rat podocytes showed an increased tendency of SCAI expression in diabetes, we examined a mouse podocyte cell line under hyperglycaemic conditions. Podocytes showed 20% increase of SCAI mRNA expression under hyperglycaemic (25 mmol/l) conditions after 48 hours, and then 40% more after 7 days, in comparison to podocytes under normoglycaemic (5 mmol/l) conditions.

Summarizing the results, healthy kidneys showed significant mRNA and protein SCAI expression. Diabetes caused a decrease in renal SCAI gene expression and protein levels in tubular cells. In contrast, both early and later effect of hyperglycemia increased gene expression of SCAI in podocytes in vitro.

5 CONCLUSIONS

The pathomechanism of diabetic nephropathy is multifactorial. When the progression reaches end-stage renal failure, quality of life and life expectancy of the patients dramatically worsen. Patients are subjected to lifelong renal replacement therapy, dialysis or transplantation, which ultimately determines the patients' life, alongside the co-existing diseases. Prevention of nephropathy, or slowing the progression with new more effective solutions may therefore be important to improve quality of life, and would cause epidemiological and economical benefits.

Based on our experiments and literature data, the increased PDE5 activity may play an important role in the progression of diabetic nephropathy. We are the first to describe, that selective inhibition of PDE5 showed protective effect on podocytes under diabetic conditions in rats by increasing cGMP levels. Better understanding of molecular mechanisms in healthy or damaged podocytes ongoing may contribute to effective treatment of proteinuria and progressive renal diseases. Results of our experiment suggest that it may be worth to test clinical applicability of selective PDE5 inhibitors as adjunctive therapy of diabetic kidney disease.

The previously unknown SCAI protein expression was also investigated in healthy and diabetic kidney in vivo and in vitro. In our experiment, healthy kidney showed significant SCAI mRNA and protein expression, and decreased SCAI expression in the diabetic whole kidney, decreased SCAI expression in tubular cells, but increased SCAI expression in podocytes. The SCAI expression decreased in α-SMA productive tubular cells, which can be associated with the development of myofibroblasts. It would be worthwhile to examine whether the stimulation of SCAI expression in diabetes would reduce EMT formation, or may provide a kind of endogenous protection against renal fibrosis.

6 BIBLIOGRAPHY OF PUBLICATIONS

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