Renal $\text{H}_2\text{S}$-producing enzymes in STZ-induced diabetes and hyperglycemic memory

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Abstract

Diabetic nephropathy is the leading cause of chronic renal failure in the Western world, and a major clinical and financial health burden. Strict glycemic control can reduce the development of micro- and macrovascular complications, including diabetic nephropathy, and the inverse holds true for dysregulation of glycemic control. Hyperglycemic stress persists, even when glucose is normalized. This phenomenon is referred to as “hyperglycemic memory”. Bioavailability of gasotransmitter hydrogen sulfide (H$_2$S) is lowered in patients with diabetes. Additionally, exogenous administration of H$_2$S is protective in (diabetic) renal disease. The effect of hyperglycemic memory on the expression of H$_2$S-producing enzymes after reversing the diabetic state into normoglycemia is as yet unkown. The aim of the present study is to determine renal H$_2$S-producing enzyme expression in the diabetic state and hyperglycemic memory conditions, and whether renal H$_2$S production is associated with progress of diabetic kidney disease. To investigate this, expression of H$_2$S-producing enzymes was studied in kidneys of streptozotocin (STZ)-induced diabetic mice, and subsequent reversal of diabetes by isogenic pancreatic islet transplantation.

Glomerulosclerosis and interstitial inflammation were studied, as markers for renal damage after STZ-induced diabetes. Renal expression of H$_2$S-producing enzymes were evaluated both at mRNA and protein level.

STZ-induced diabetes induced renal damage, as evidenced by increased glomerular αSMA-positivity, reduced podocin expression, and increased influx of interstitial macrophages. The expression of renal H$_2$S-producing enzymes at mRNA level was not significantly altered by induction of diabetes. At protein level, renal CBS expression was drastically reduced after STZ-induced diabetes, which was partly restored after islet transplantation. Diabetes-induced renal expression of CSE en 3MST were less pronounced, although also renal CSE protein expression was lower in 6-weeks diabetic mice, which was not restored after islet transplantation.

To conclude, STZ-induced diabetes profoundly reduced renal CBS expression. Reversal of diabetes by isogenic pancreatic islet transplantation normalized renal CBS protein levels but did not improve renal damage markers. This suggests that renal CBS expression can be partly dependent on glucose or insulin levels, and that restored CBS expression is not able to restore diabetes-induced renal damage. This implicates that CBS would not be a therapeutic target to reduce diabetic kidney injury.
Introduction

Diabetes mellitus is characterized by hyperglycemia and insulin deficiency (type 1) or resistance (type 2). Diabetes is a worldwide health burden; its prevalence is increasing up to epidemic proportions and currently estimated to be 9% among adults. Both type 1 and type 2 diabetes are important risk factors for cardiovascular diseases, with a 2- to 4-fold increased risk when compared to non-diabetic individuals. Moreover, based on US data, diabetic kidney disease (i.e. diabetic nephropathy) is the leading cause of chronic renal failure in the Western world, accounting for about 40% of new cases of end stage renal disease. Clinically, diabetic nephropathy is accompanied by proteinuria and chronic renal failure. At histological level, both glomeruli and interstitium are affected.

Several large clinical trial on type 1 and type 2 diabetes have demonstrated that strict glycemic control can reduce the development of micro- and macrovascular complications. Interestingly, the advantageous effects of strict glycemic control persist in the long term (i.e. also in the presence of later less strict glycemic control). Even several years after the trials ended, subjects from the intensive glycemic control arm of the study continued to have a deceased risk for developing renal impairment and cardiovascular disease. The Diabetes Control and Complications Trial and Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) collaboration group first described this phenomenon as “metabolic memory”. Interestingly, also the reciproce holds true for dysregulation of glycemic control. Previous research demonstrated that hyperglycemic stress in the past persists despite normalized glycemic control. This phenomenon is described as “hyperglycemic memory”.

Hydrogen sulfide ($H_2S$) is a small gaseous signaling molecule which is endogenously produced. $H_2S$ is member of the gasotransmitter family, which also includes nitric oxide (NO) and carbon monoxide (CO). $H_2S$ is endogenously produced by three different enzymes in the mammalian body: the two pyridoxal-5'-phosphate (PLP)-dependent enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3MST). $H_2S$ is physiologically active, causing vasodilation, inducing angiogenesis, and scavenging of reactive oxygen species (ROS).

In the diabetic state, the role of $H_2S$ is still controversial though. In human subjects with diabetes mellitus, $H_2S$ bioavailability is lower compared to non-diabetic healthy subjects. In non-obese type 1 diabetic mice $H_2S$ levels are lower compared to non-diabetic control mice despite higher vascular levels of CSE and CBS.
explain this contradiction as compensatory response upon lower \( \text{H}_2\text{S} \)-bioavailability, but it shows that measured patterns of \( \text{H}_2\text{S} \) levels and expression of \( \text{H}_2\text{S} \)-producing enzymes do not a priori correspond. In spontaneous diabetic Ins2\(^{Akita} \) mice, renal CSE and CBS were considerably lowered compared to healthy control mice, which was accompanied by lower \( \text{H}_2\text{S} \) production levels in the kidney.\(^{22} \) Also in streptozotocin (STZ)-induced type 1 diabetes male Sprague–Dawley rats, lower plasma levels of \( \text{H}_2\text{S} \) were observed compared to non-diabetic littermates.\(^{19} \) Exogenous administration of \( \text{H}_2\text{S} \) donor NaHS prevented progression of diabetic renal damage in STZ-induced diabetes in rats.\(^{23,24} \) On the contrary, CSE deficiency significantly delayed the development of STZ-induced type 1 diabetes, which was accompanied by lower levels of apoptotic \( \beta \)-cells.\(^{25} \) \( \text{H}_2\text{S} \) was found to be involved in glucose synthesis in hepatocytes \textit{in vitro},\(^{26} \) and CSE-deficient mice showed decreased gluconeogenesis with concomitant lower levels of plasma glucose.\(^{27} \)

Taken together, the role of \( \text{H}_2\text{S} \) in diabetes and diabetic renal disease is not completely understood and it is of interest to investigate whether renal \( \text{H}_2\text{S} \) production is associated with progression of diabetic kidney disease.

It is reported before that insulin treatment could normalize diabetes-induced changes in \( \text{H}_2\text{S} \)-producing enzymes.\(^{28} \) However, it is unknown what occurs to the expression of \( \text{H}_2\text{S} \)-producing enzymes when the diabetic state is completely reversed, and animals are strictly normoglycemic after a period of hyperglycemia. Therefore, the aim of the present study is to determine renal \( \text{H}_2\text{S} \)-producing enzymes in the diabetic state and hyperglycemic memory conditions. To investigate this, \( \text{H}_2\text{S} \)-producing enzymes, both at mRNA and protein level, were studied in kidneys of STZ-induced diabetic mice, and subsequent reversal towards normoglycemia by isogenic pancreatic islet transplantation.

**Material and Methods**

**Animals**

Male C57Bl/6J mice (Charles River, Germany) were housed with a 12-/12-h light/dark cycle with \textit{ad libitum} access to standard rodent chow and water. All procedures were in agreement with institutional and legislator regulations and approved by the Committees on the Ethics of Animal Experiments of the Universities of Giessen and Heidelberg/ Mannheim.
**Experimental groups**

The experiment consists of 4 experimental groups (Figure 1). Group 1, healthy control mice (Control); group 2, STZ-induced diabetic mice, terminated after 6 weeks of diabetes induction (6W DM); group 3, STZ-induced diabetic mice which received isogenic pancreatic islet transplantation after 6 weeks of diabetes induction and terminated 6 weeks after islets transplantation (Islets Tx); group 4, STZ-induced diabetic mice, terminated after 12 weeks of diabetes induction (12W DM).

![Experimental groups diagram](image)

**Diabetes induction by streptozotocin**

Streptozotocin (STZ) (Sigma-Aldrich) was freshly prepared in 0.05 M citratebuffer, pH 4.4 – 4.6, and was administered once to 8-week-old C57Bl/6J mice at a dose of 160 mg/kg via intraperitoneal injection. Blood glucose levels were measured regularly via venous blood sampling from the tail. Diabetes was defined as a sustained elevation of fasting blood glucose >14 mmol/l.
Islet isolation and transplantation

Islet isolation and transplantation was performed in the University of Giessen. Islets were isolated from 8-week-old male C57Bl/6J mice (Janvier Labs, France). Pancreata were dissected, minced, and subsequently incubated with 1.6 to 2.0 mg/mL collagenase in HBSS in a shaking water bath at 37°C. Islets were handpicked and subsequently suspended in CMRL-1066 Medium supplemented with 10% FBS, 100µg/mL streptomycin, and 20 µg/mL ciprofloxacin. Free-floating pancreatic islets were cultered overnight under standardized conditions, and tested for viability with the propidium iodide assay and by glucose stimulated insulin secretion. Recipient mice were anesthetized, and the kidney was assessed by an incision of the left flank. Pancreatic islets were transplanted under the left kidney capsule of recipient C57Bl/6J mice on week 6 after induction of diabetes. To ensure normoglycemia, islet transplanted mice also received subcutaneous insulin pellets (Linbit, LinShin Canada), that slowly release insulin over time (~0.1 U/24 hr/implant). The amount of insulin releasing pellets was determined by blood glucose levels (<14 mmol/l). All animals were terminated under general anesthesia, blood and kidneys were collected and snap-frozen for biochemical and histological analysis.

Polymerase Chain Reaction

RNA was isolated from frozen kidney samples using TRIzol Reagent (Invitrogen). The RNA concentrations were measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). 1 ug RNA was converted into cDNA using SuperScript II reverse transcriptase and random hexamere primers (Life Technologies). CSE (Cth) (assay Mm00461247_m1), CBS (assay Mm00460654_m1), 3MST (Mpst) (assay Mm00460389_m1), and Podocin (Nphs2) (assay Mm01292252_m1) mRNA expression was measured with Taqman Gene expression assays (Applied Biosystems). For normalization, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ (Ywhaz) (assay Mm03950126_s1) was included as housekeeping gene. Reactions were performed on an ABI7900HT thermal cycler (Applied Biosystems). The comparative Ct method ($2^{-\Delta\Delta C_t}$ method) was used to calculate relative gene expression.

Immunohistochemical Stainings

Frozen sections of 4 µm were stained with PAS staining to assess general morphology. For immunohistochemistry, frozen sections were fixed for 10 minutes in acetone. Sections were subsequently stained for αSMA (mouse monoclonal anti-αSMA, clone ASM-1, ProGen),
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F4/80 (rat anti F4/80, clone BM8 (ab16911), Abcam), or podocin (rabbit polyclonal anti-Podocin, clone P0372 Sigma). Following incubation with primary antibodies (1 hr, room temperature), sections were incubated with HRP-conjugated rabbit-anti-mouse IgG (αSMA), rabbit-anti-rat IgG (F4/80), or goat-anti-rabbit IgG (Podocin) polyclonal antibodies (Dako). 3-amino-9-ethylcarbazole (AEC) was used as chromogen, and nuclei were counterstained using hematoxylin after which the sections were coverslipped.

**Western Blot**

Protein was isolated from mouse kidney cryosections (30x10µm) using RIPA Buffer supplemented with 1% Halt Phosphatase inhibitor cocktail and 1% Halt Protease inhibitor cocktail. Total protein content of the lysates was determined with the pyrogallol red method as previously described. Briefly, 5 µl of sample was added in a 96-well plate (Corning), after which 300 µl pyrogallol red-molybdate complex was added. After 10 minutes incubation, absorbance was measured at 600 nm (Varioskan, Thermo Scientific) and protein concentration was calculated against a calibration curve. Equal amounts of protein (20 µg) were boiled and electrophoretically separated in 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Aspecific binding of antibodies was blocked using 5% non-fat milk powder in Tris-buffered saline-0.1% Tween-20 (TBST) for 1 hour. Membranes were incubated with primary antibody overnight at 4°C. The following antibodies were used: CSE; 1:2000, mouse monoclonal IgG1κ, Abnova clone M03), CBS; 1:500, (polyclonal rabbit anti-CBS, clone AV45746, Sigma), 3MST; 1:2000 (polyclonal rabbit anti-MPST, clone HPA001240, Sigma), and Podocin; 1:500 (polyclonal rabbit anti-Podocin, clone P0372, Sigma). Appropriate horseradish peroxidase-labeled antibody was used as a detection antibody. HRP-conjugated β-actin; (1:5000, monoclonal mouse anti-β-actin, clone sc-47778 HRP, Santa Cruz Biotechnology), served as housekeeping protein. All antibody incubations were followed by washing with TBST. Immunoreactivity was visualized by ECL Western Blotting Substrate (Thermo scientific, Waltham, MA, USA), and images were taken with the Bio-Rad-ChemiDoc MP system and quantified using ImageLab software.

**Statistical analyses**

All data were analyzed with GraphPad PRISM 5.0 (GraphPad, San Diego, CA, USA). Normality was tested using the Kolmogorov–Smirnov test. Normally distributed data were analyzed using one-way ANOVA, and non-parametric distributed data were analyzed using Kruskal Wallis test. Bonferroni (one-way ANOVA) or Dunns (Kruskal Wallis) post-hoc analysis
was applied to correct for multiple comparisons. Data are shown as mean±SEM (Standard Error of the Mean). Differences were considered statistically significant when $P<0.05$.

Results

Glucose levels and body weight
After β-cell destruction with STZ, glucose levels increased to levels above the detection limit (>33.3 mmol/L) in 6 week and 12 week diabetic animals. In healthy mice, mean (±SD) blood glucose was 11.2 ± 1.5 mmol/L. Islet transplantation lowered blood glucose with 78% to 7.3 ± 1.2 mmol/L ($P<0.01$). Blood glucose over time for the islet transplanted group is shown in Figure 2. Healthy control mice, 12 weeks after start of the study, weighed 32.3 ± 3.1 gram. Diabetic mice had a lower body weight of 23.2 ± 1.3 gram after 6 weeks, and 23.6 ± 2.3 gram after 12 weeks of diabetes ($P<0.05$). Islet transplantation significantly increased body weight compared to 6 weeks and 12 weeks diabetic mice (33.2 ± 1.3 gram; $P<0.05$). Glucose levels and body weight data of all the groups at the time of termination is depicted in Table 1.

STZ-induced diabetes results in glomerular injury
Glomerulosclerosis, as measured by αSMA-positivity within the glomerulus, was increased in STZ-induced diabetic mice. 6 weeks ($P<0.05$) and 12-weeks diabetic mice ($P<0.01$) had a 4-fold increase in the number of affected glomeruli compared to healthy controls. This glomerular damage was not attenuated after isogenic transplantation of pancreatic islets as evident from the same number of affected glomeruli in the islet Tx group compared to the 12-weeks diabetic mice (Figure 3A). To investigate podocyte injury as a hallmark of diabetic kidney disease, podocin expression was analyzed. Podocin protein expression was visualized by immunohistochemistry and quantified by western blot analysis. Podocin expression was significantly lower in diabetic mice (both at 6 and 12 weeks diabetes), which was not altered by islet transplantation (Figure 3B). In the serial sections used for western blot analysis, glomeruli were counted. The number of counted glomeruli was not different across the groups, indicating that the number of glomeruli per cross section does not account for the difference in podocin expression as determined by western blot analysis (Supplemental figure 1). Diabetes did not significantly change renal function, measured by serum creatinine and blood urea nitrogen (Supplemental figure 2).
Table 1 - Blood glucose and body weight at time of termination

<table>
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<th>12W DM</th>
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<tr>
<td>Glucose (mmol/L)</td>
<td>11.2 ± 1.5</td>
<td>&gt;33.3 ± 0</td>
<td>7.3 ± 1.7 **##</td>
<td>&gt;33.3 ± 0</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>32.4 ± 3.1</td>
<td>23.2 ± 1.3</td>
<td>33.2 ± 1.3 **</td>
<td>23.6 ± 2.3 $^5$</td>
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$^5 P<0.05$ vs. Control; * $P<0.05$ vs. 6W DM; ** $P<0.01$ vs. 6W DM; $^# P<0.05$ vs. 12W DM; ## $P<0.01$ vs. 12W DM

Figure 2 - Blood glucose is drastically lowered by islet transplantation. Six weeks after STZ-induced diabetes, isogenic pancreatic islet transplantation was performed, in combination with insulin treatment, resulting in a reduction of blood glucose to physiological levels.

Interstitial inflammation is increased in kidneys of STZ-induced diabetic mice

Macrophages were visualized and quantified by F4/80 immunohistochemistry. Macrophages were present in the interstitium, but not within the glomeruli. The number of macrophages was significantly increased by induction of diabetes. Compared to healthy controls, in 6-weeks and 12-weeks diabetic mice, interstitial macrophages were increased with 113% and 138% respectively ($P<0.05$). The number of macrophages was not significantly altered after islet transplantation, and remained significantly increased compared to healthy control ($P<0.05$, Figure 4).
Figure 3 - STZ-induced diabetes resulted in glomerular injury. STZ-induced diabetes resulted in increased glomerular injury as shown by increased αSMA positivity within the glomerulus (A) and decreased renal podocin expression (B). *P< 0.05 vs. control; ** P<0.01 vs. control; *** P<0.001 vs. control. Bar indicates 60 µm.

Figure 4 - Interstitial inflammation in increased after STZ-induced diabetes. Number of interstitial macrophages were more than two-fold increased after STZ-induced diabetes in mice. No macrophages were present in the glomeruli, as shown in the high power inset. F4/80+ macrophages were quantified in 20 high power fields (HPF) at 40x magnification. *P< 0.05 vs. non-diabetic control. Bar indicates 100 µm.
**Renal mRNA levels of H₂S producing enzymes**

Renal mRNA expression levels of H₂S-producing enzymes were not significantly altered by STZ-induced diabetes (Figure 5). Despite the fact that no significant differences were observed, there was a tendency of higher expression of CSE (A) and 3MST (C), and lower expression of CBS (B) after STZ-induced diabetes. These changes were normalized after transplantation of isogenic pancreatic islets.

![Figure 5](image)

**Figure 5** - Renal mRNA expression levels of H₂S-producing enzymes. Renal mRNA expression levels of H₂S-producing enzymes were not significantly altered by STZ-induced diabetes. Although, there was a tendency of higher expression of CSE (A) and 3MST (C), and lower expression of CBS (B) after STZ-induced diabetes. These changes were normalized after transplantation of isogenic pancreatic islets.

**Renal CSE and CBS protein expression levels are decreased after induction of diabetes**

Representative results (of 2 mice/group) from renal protein expression levels of H₂S-producing enzymes, as quantified by western blot analysis, are shown in Figure 6A. CBS expression was drastically reduced after STZ-induced diabetes, which was partly but significantly restored after islet transplantation (Figure 6B). Renal CSE expression in 6-weeks diabetic mice was reduced compared to healthy controls. However, this reduction of CSE expression was slightly restored after 12 weeks of diabetes (Figure 6C). 3MST expression in the kidney was slightly upregulated in 12-weeks diabetic mice, and significantly higher than renal 3MST expression is mice from the islets Tx group, indicating that islet transplantation prevents this increase in 3MST expression (Figure 6D).
Figure 6 - renal protein expression levels of H₂S-producing enzymes. Representative western blots for H₂S-producing enzymes among the different groups are shown in panel A. CBS expression was more than 80% reduced after STZ-induced diabetes, which was partly normalized after islet transplantation (B). Renal CSE expression in 6-weeks diabetic mice was reduced compared to healthy controls. However, this reduction of CSE expression did not last until 12 weeks of diabetes (C). 3MST expression in the kidney was slightly upregulated in 12-weeks diabetic mice, but no significant differences were observed before that stage (D).
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Discussion

In this study, we report that STZ-induced diabetes in mice is associated with reduced renal expression of CBS. This reduction was altered after reversal of the diabetic, hyperglycemic state with islet transplantation in combination with insulin treatment. Changes in renal expression of CSE and 3MST were less pronounced, although also renal CSE protein expression was lower in 6-weeks diabetic mice, which was not restored after islet transplantation. Diabetes increased renal 3MST expression after 12 weeks (but not after 6 weeks) of diabetes. To our best knowledge, this is the first report on renal H₂S-producing enzymes after STZ-induced diabetes and its associations with hyperglycemic memory.

Our data are in line with previous reports which demonstrated lower bioavailability of H₂S, and downregulated renal expression levels of H₂S-producing enzymes in the diabetic state. It is reported before that insulin treatment could normalize diabetes-induced changes in H₂S-producing enzymes in liver and pancreas. However, in this particular study, STZ-induced diabetic rats still suffered from significant hyperglycemia after insulin treatment (mean glucose 17 ± 4 mmol/L), and no renal expression levels of H₂S-producing enzymes were measured.

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease, and especially in patients with diabetes. CBS and CSE are both involved in the transsulfuration pathway, where they both degrade homocysteine and cysteine to form H₂S. Interestingly, elevated plasma homocysteine levels in human diabetic patients appear to depend on the presence of diabetic kidney disease rather than just diabetes. Diabetic patients with impaired renal function tend to have increased homocysteine level, in contrast to diabetic patients without diabetic kidney failure. These data emphasize the importance of the kidney in the transsulfuration pathway and the apparent central role of the kidney in circulating homocysteine levels.

The renal damage in this model was mild as indicated by the slight decrease in podocin levels and no significant changes in serum creatinine. The possible explanation could be the short follow-up of 12 weeks. It could well be that a longer follow-up is needed to study true diabetic nephropathy. However, we are interested in mild diabetes-related renal damage instead of full-blown diabetic nephropathy, as we believe that this mild renal damage is representative for a large group of patients with diabetes and poor glycemic control, but without overt diabetic nephropathy. The lack of H₂S measurements can be regarded as limitation of this study. However, the different H₂S measurements
are still in the developmental stage, not without controversy, and there is no consensus on the physiological levels of H$_2$S in the blood. Therefore, we only measured H$_2$S-producing enzymes. The discrepancy between renal mRNA and protein expression levels was an unexpected finding, but might be related to a high protein turnover in the diabetic state. Future studies to analyze the potential effects of diabetes on transcriptional and translational regulation of enzymes involved in H$_2$S biosynthesis are therefore warranted. Changes in H$_2$S-producing enzymes upon diabetic challenge are organ dependent. As earlier described, STZ-induced diabetes resulted in upregulated levels of hepatic and pancreatic CSE and CBS, but renal H$_2$S-producing activity and plasma H$_2$S levels were slightly (not significantly) lower. This report again suggests that the kidney contributes significantly to the plasma H$_2$S levels.

Hyperglycemic memory on renal damage was not observed since renal damage did not progress after 6 weeks of STZ-induced diabetes. Islet transplantation did not reverse diabetic renal damage, but rather stabilizes it. However, in 12-weeks diabetic mice, renal damage was roughly similar to 6-weeks diabetic mice, indicating that the vast majority of renal damage already emerged within the first 6 weeks after induction of diabetes. Renal CBS expression was attenuated by islet transplantation in contrast to renal CSE expression. This indicates that CBS expression, and not CSE expression could be dependent on glucose or insulin levels. Higher renal expression of CBS were not accompanied by less damage in mice from the islet Tx group, indicating that restored H$_2$S bioavailability is not able to restore diabetes-induced renal damage. This implicates that CBS would not be a therapeutic target to reduce diabetic kidney injury.

To conclude, renal expression of CSE and especially CBS is lowered in STZ-induced diabetic mice. Reversal of diabetes by isogenic pancreatic islet transplantation normalized CBS levels but did not improve renal damage markers. This indicates that renal CBS expression can be partly dependent on glucose or insulin levels. Additionally, higher CBS levels were not accompanied by less damage in islet Tx mice, indicating that restored H$_2$S bioavailability is not able to restore diabetes-induced renal damage. Future long-term follow-up studies are needed to examine this premise.

**Acknowledgements**

We thank Sippie Huitema for her excellent technical assistance.
Supplemental Figures

Supplemental figure 1 - Number of glomeruli does not differ between groups. Number of glomeruli was counted per cross section. No significant differences were observed between groups.

Supplemental figure 2 - No significant differences in renal function between healthy control and 12-weeks diabetic mice. STZ-induced diabetes did not significantly change renal function although a tendency of higher serum creatinine (P=0.55) and blood urea nitrogen (P=0.31) levels in 12-weeks diabetic mice compared to healthy controls was observed.
References


