ASK1 inhibitor halts progression of diabetic nephropathy in Nos3 deficient mice.

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Running Title: ASK1 in diabetic nephropathy

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ABSTRACT

p38 mitogen-activated protein kinase (MAPK) signaling promotes diabetic kidney injury. Apoptosis signal-regulating kinase-1 (ASK1) is one of the upstream kinases in the p38 MAPK signaling pathway which is activated by inflammation and oxidative stress, suggesting a possible role for ASK1 in diabetic nephropathy. In this study, we examined whether a selective ASK1 inhibitor can prevent the induction and progression of diabetic nephropathy in mice. Diabetes was induced in hypertensive endothelial nitric oxide synthase (Nos3) deficient mice by 5 low-dose streptozotocin (STZ) injections. Groups of diabetic Nos3-/− mice received ASK1 inhibitor (GS-444217 delivered in chow) as an early intervention (2-8 weeks after STZ) or late intervention (weeks 8-15 after STZ). Control diabetic and non-diabetic Nos3-/− mice received normal chow. Treatment with GS-444217 abrogated p38 MAPK activation in diabetic kidneys, but had no effect upon hypertension in Nos3-/− mice. Early intervention with GS-444217 significantly inhibited diabetic glomerulosclerosis and reduced renal dysfunction, but had no effect on the development of albuminuria. Late intervention with GS-444217 improved renal function and halted the progression of glomerulosclerosis, renal inflammation and tubular injury despite having no effect on established albuminuria. In conclusion, this study identifies ASK1 as a new therapeutic target in diabetic nephropathy to reduce renal inflammation and fibrosis independent of blood pressure control.
KEYWORDS

apoptosis signal-regulating kinase-1 (ASK-1)
diabetic nephropathy
p38 MAPK
glomerulosclerosis
inflammation
renal function

ABBREVIATIONS

ACR  albumin:creatinine ratio
ASK1  apoptosis signal-regulating kinase1
NOS3  endothelial nitric oxide synthase
MAPK  mitogen-activated protein kinase
MCP-1  monocyte chemoattractant protein-1
STZ  streptozotocin
INTRODUCTION

Diabetic nephropathy is the most common single cause of end-stage renal failure in many countries. Current therapies of controlling blood pressure and blood glucose levels have only a limited benefit in slowing progression to end-stage disease (1), indicating a major gap in our treatment options. The diabetic kidney is stressed by multiple factors including hyperglycemia, reactive oxygen species, advanced glycation end-products, pro-inflammatory cytokines and angiotensin II – all of which can induce signaling via the p38 mitogen-activated protein kinase (MAPK) (2-5). Increased activation of p38 MAPK in glomeruli and the tubulointerstitial compartment has been described in animal models of diabetic nephropathy as well as in patients with diabetic nephropathy (6-9). Inhibition of p38 MAPK activation via pharmacologic and genetic approaches can suppress the induction of albuminuria, glomerular matrix expansion and inflammation in models of type 1 and type 2 diabetic nephropathy (9; 10), although intervention studies in established disease are lacking. Importantly, clinical trials of p38 inhibitor compounds in rheumatoid arthritis have failed to deliver the promise of animal studies due to toxicity issues, which have led to investigation of the upstream kinases involved in context-dependent p38 MAPK activation (11).

Apoptosis signal-regulating kinase 1 (ASK1/MAP3K5) is a member of the large family of MAPKKK enzymes, many of which have the potential to activate the downstream p38 MAPK via phosphorylation of the MKK3 and MKK6 enzymes (12; 13). p38 MAPK and, to a lesser extent c-Jun terminal kinase, are the only known downstream targets of ASK1 signalling (14). ASK1 is activated in response to oxidative stress; specifically ASK1 exists as an inactive dimer coupled to thioredoxin and undergoes auto-activation following the oxidation and dissociation of thioredoxin (13). ASK1 is widely expressed in diverse tissues, and is most highly expressed in the kidney (15). Mice lacking the Ask1 gene (Ask1/-) have a normal phenotype, including normal kidney structure and function, which contrasts with the foetal lethality of mice lacking the p38α/Mapk14 gene (16; 17). In addition, Ask1/- mice show a dramatic inhibition of p38 MAPK activation and significant
protection from kidney injury in models of acute tubular necrosis and renal interstitial fibrosis, providing results consistent with administration of p38 inhibitors (4; 18-20). Therefore, inhibition of ASK1/p38 signaling may have therapeutic potential for diabetic glomerulosclerosis.

This aim of this study was to investigate the therapeutic potential of a highly selective small molecule ASK1 inhibitor (GS-444217) in experimental diabetic nephropathy. We investigated streptozotocin-induced type 1 diabetes in hypertensive mice lacking the gene for endothelial nitric oxide synthase (Nos3) as this model exhibits robust development of diabetic glomerulosclerosis (21).

MATERIALS AND METHODS

ASK1 Inhibitor

GS-444217 was synthesized by Gilead in Foster City, CA (manuscript in preparation). In a competitive, time-resolved fluorescence resonance energy transfer immunoassay, GS-444217 directly inhibited ASK1 kinase activity in vitro with an IC\textsubscript{50} of 2.9 ± 0.8nM. In a kinase selectivity panel that included 451 kinases (KINOMEscan®, DiscoverRX Corporation, Fremont, CA), GS-444217 exhibited over 50-fold greater affinity for ASK1 compared to all other kinases measured. For delivery in mice, GS-444217 was incorporated into standard mouse chow at 0.1% (wt/wt). The concentration of GS-444217 in mouse plasma was measured at the end of each study and found to be 20.2 ± 8.2 µM for the early intervention study and 21.3 µM ±7.4 µM for the late intervention study. These exposures of GS-444217 in mice are expected to result in complete and selective inhibition of ASK1 (data not shown).

Animal Model of Diabetic Nephropathy

Nos3−/− mice on the C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and bred under pathogen-free conditions at the Monash Medical Centre Animal Facility
(Clayton, Australia). At 8 weeks of age, male Nos3-/- mice were given intraperitoneal injections of streptozotocin (STZ; 5 x 55mg/kg/day; Sigma, St Louis, MO, USA). Groups of mice (n=10) with diabetes (fasting blood glucose >16 mmol/l at 2 weeks after the last STZ injection) were followed for 8 or 15 weeks. In the early intervention study, diabetic mice were fed either normal chow or chow containing 0.1% GS-444217 between weeks 2 and 8 and then killed. In the late intervention study, diabetic mice were fed either normal chow or chow with 0.1% GS-444217 over weeks 8 to 15 and then killed. Age-matched non-diabetic Nos3-/- mice (n=10) were used as controls. In an additional study, a group of non-diabetic Nos3-/- mice (n=9) were assessed for systolic blood pressure before and after a 6 week period on chow containing 0.1% GS-444217. GS-444217 treatment was well tolerated by the mice in all studies and no drug-related clinical observations were noted in any of the studies. Blood glucose (measured by a tail vein sample) and body weight were measured weekly after a 3 hour fast (0800-1100) and mice with fasting glucose levels >30mmol/l were given 0.5 units of protophane insulin (Novo Nordisk, Sydney, Australia) subcutaneously three times a week to maintain body weight. Urine was collected pre-experiment and at weeks 5, 8, 12 and 15 after STZ injection to assess urine albumin excretion. Glycated hemoglobin (HbA1c) was measured from blood samples taken at weeks 8 and 15. Tissues were collected at week 8 or 15 and were fixed in 4% (vol/vol) formaldehyde, 2% (wt/vol) paraformaldehyde-lysine-periodate (PLP), or snap-frozen and stored at -80°C. These animal studies were approved by the Monash Medical Centre Animal Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition (2004).

**Biochemistry**

Fasting blood glucose was measured from tail blood by glucometer (Medisense, Abbott Laboratories, Bedford, MA). Urine was collected from mice housed in metabolic cages for 8 hours. Heparinized whole blood was collected from tail veins for analysis of HbA1c. At the end of
experimentation, whole blood was collected by cardiac puncture in anaesthetized animals and stored as serum or heparinized plasma. Urine creatinine levels were determined by the Jaffe rate reaction method. ELISA kits were used to assess levels of urine albumin (Bethyl Laboratories, Montgomery, TX, USA), plasma insulin (Mercodia, Uppsala, Sweden) and serum cystatin-C (Enzo Life Sciences, Farmingdale, NY, USA). HbA₁c was measured by DCA Vantage Analyzer (Siemens, Camberley, UK).

**Blood Pressure Analysis**

Systolic blood pressure (SBP) was measured in conscious mice by tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA, USA). Mice were trained twice weekly for 3 weeks prior to experimental readings. At each recording, mice were acclimatised to a preheated chamber (29°C) for 15 min and the pressure readings were recorded over 3 consecutive manual inflation-deflation cycles to obtain an average.

**Antibodies**

The following primary antibodies were used in this study: mouse anti-phospho-p38α (Upstate Biotechnology, Lake Placid, NY, USA); rat-anti CD68 (FA-11; Serotec, Oxford, UK); goat anti-collagen IV (Southern Biotechnology, Birmingham, AL); and rabbit anti-Wilm’s Tumour 1 (WT1) antigen (Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunohistochemistry**

Formalin-fixed sections (2µm) were stained with Periodic acid-Schiff (PAS) to assess structure and counterstained with hematoxylin to identify nuclei. Immunostaining for phospho-p38 and collagen IV was performed on 4µm paraffin embedded sections which were fixed in 4% formalin or methylcarn solution. Immunostaining for CD68 was performed on 5µm PLP-fixed cryostat sections. For antigen retrieval of phospho-p38, dewaxed paraffin sections were heated in a microwave oven
(800W, 12 min) in 10 mmol/l sodium citrate buffer (pH 6.0) (22). For immunostaining, sections were treated with 20% rabbit serum or 20% goat serum for 30 min and then incubated with primary antibody in 3% BSA overnight at 4°C. Sections were then placed in 0.6% hydrogen peroxide in methanol for 20 min to inactivate endogenous peroxidase. Bound primary antibodies were detected using a standard ABC-peroxidase system: avidin-biotin block, biotinylated antibodies (rabbit anti-goat IgG, rabbit anti-rat IgG, or goat anti-mouse IgG) and ABC-peroxidase (Vector Laboratories, Burlingame, CA, USA). Sections were developed with 3,3-diaminobenzidine (Sigma) to produce a brown colour. CD68 sections were counterstained with hematoxylin to assist cell counting. Normal goat serum or isotype-matched irrelevant IgG were used as negative controls.

**Quantitation of Immunohistochemistry**

The number of CD68+ cells and WT-1+ podocytes was counted in 30 hilar glomerular cross-sections (gcs) per animal (x400). Glomerular collagen IV staining was quantitated by computer image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA) in 50 hilar gcs (x400), and expressed as the percentage of glomerular area stained. All scoring was performed on blinded slides.

**Real-time PCR**

Total RNA was extracted from whole kidney using Trizol (Invitrogen) and reverse transcribed with random primers using the Superscript First-Strand Synthesis kit (Invitrogen). Real-time PCR analysis was performed as previously described (5; 23). The Taqman probe and primer sequences are listed in Supplementary Table 1.
Statistical Analysis

Statistical differences were analysed by one way ANOVA with Tukey’s multiple comparison post-test. Data were recorded as mean ± SEM with \( p<0.05 \) considered significant. All analyses were performed using GraphPad Prism 6.0 (GraphPad software, San Diego, CA, USA).

RESULTS

Effect of ASK1 inhibitor on p38 MAPK signaling and blood pressure

Consistent with previous studies (6; 9; 24), basal p38 phosphorylation (activation) was detected in non-diabetic kidney by Western blot while immunostaining identified phosphorylated p38 in glomeruli, tubules and the interstitium (Fig. 1A and E). A significant increase in p38 activation in the kidney was evident at both weeks 8 and 15 (Fig. 1). In contrast, treatment with GSU444217 over weeks 2 to 8 and over weeks 8 to 15 of diabetes caused a dramatic inhibition of p38 activation in all cell types (Fig. 1).

Since diabetes does not affect hypertension in Nos3--/-- mice (25; 26), we measured the effect of GSU444217 on blood pressure in non-diabetic Nos3--/-- mice. Systolic blood pressure in Nos3--/-- mice was 133±5mmHg before treatment and was unchanged (134±5mmHg) after 6 weeks of GSU444217 administration, demonstrating that GSU444217 does not affect hypertension in these mice.

Early intervention with GS-444217 inhibits the development of diabetic renal injury

Groups of diabetic Nos3--/-- mice were fed standard chow or chow with 0.1% GS-444217 from week 2 after STZ injection until being killed on week 8. GS-444217 had no effect upon fasting body weight, fasting blood glucose levels or plasma insulin levels, although a partial reduction in HbA1c levels was evident in the drug treated group (Fig. 2). A substantial increase in urinary albumin excretion was evident on weeks 5 and 8 after STZ injection which was not affected by GS-444217 treatment (Fig. 3A). There was also a reduction in the number of glomerular WT1+ podocytes at
week 8 after STZ, which was unaffected by drug treatment (Fig. 3B). However, the decline in renal function at week 8 after STZ, as assessed by increased serum cystatin C levels, was significantly improved by drug treatment (Fig. 3C).

*Nos3-/−* mice with no treatment developed mild to moderate glomerulosclerosis by week 8 of diabetes as shown by increased PAS-stained glomerular deposits and an increase in glomerular staining for collagen IV (Fig. 4). In addition, a significant glomerular infiltrate of CD68+ macrophages was evident (Fig. 4). GSU444217 significantly reduced both glomerulosclerosis and glomerular macrophage infiltration (Fig. 4).

**Late intervention with GS-444217 improves renal function and halts progressive glomerulosclerosis in established diabetic nephropathy**

Groups of diabetic *Nos3-/−* mice were fed standard chow or chow with 0.1% GSU444217 from week 8 after STZ injection until being killed on week 15. In animals fed standard diet, disease severity was maintained or increased between weeks 8-15 (Figs 5 and 6). GSU444217 had no effect upon fasting body weight, fasting blood glucose levels or HbA1c levels (Fig. 5). The diabetic mice had established albuminuria and raised serum cystatin-C levels at week 8 when GSU444217 treatment commenced (Fig. 6). Drug treatment had no impact upon established albuminuria or podocyte loss, but there was a significant reduction in the levels of serum Cystatin-C, suggesting that GS-444217 treatment improved renal function (Fig. 6).

There was a clear increase in diabetic glomerulosclerosis between weeks 8 and 15 in untreated diabetic *Nos3-/−* mice as shown glomerular collagen IV staining (Fig. 7A-C and E). This was associated with a sustained increase in renal mRNA levels for pro-fibrotic molecules (Collagens
and 4, fibronectin, TGF-β1 and PAI-I) (Fig. 7F-J) and a sustained glomerular CD68+ macrophage infiltrate and increases in renal mRNA levels for pro-inflammatory molecules (CCL2 and TNF-α) (Fig. 8A-F). In addition, expression of the tubular damage marker KIM-1 was elevated at week 8 of diabetes and remained elevated through to week 15 (Fig. 8G).

GS-444217 treatment over weeks 8 to 15 prevented the increase in glomerular deposition of collagen IV and down-regulated renal mRNA levels for all of the pro-fibrotic molecules analysed (Fig. 7D-J). In addition, GS-444217 treatment significantly reduced the glomerular macrophage infiltrate, reduced the renal mRNA expression of pro-inflammatory markers and tubular damage marker KIM-1 (Fig. 8).

**DISCUSSION**

Administration of an ASK1 inhibitor, GS-444217, was shown to be effective in a mouse model of established diabetic nephropathy on the basis of halting progressive glomerulosclerosis, reducing inflammation and improving renal function despite ongoing hyperglycemia, hypertension and albuminuria.

The protective effect of ASK1 inhibitor treatment in diabetic nephropathy was attributed to the highly effective blockade of p38 activation – the major downstream target for ASK1 (14). GS-444217 is highly selective for ASK1, although as in all pharmacology studies, we cannot formally rule out unanticipated off-target effects. Increased p38 activation has been described in human and experimental diabetic nephropathy (6-9), which was confirmed in the current study. Administration of a p38 MAPK inhibitor has been shown to reduce albuminuria and prevent the development of mild glomerular fibrosis in non-hypertensive diabetic rats (10). In addition, deletion of the *Mkk3* gene in *db/db* mice with type 2 diabetes significantly reduced p38 activation, albuminuria, podocyte
loss and glomerular collagen deposition, and improved renal function (9). The current study
significantly extends these findings in two ways. First, diabetic Nos3-/− mice develop a much more
severe form of glomerulosclerosis compared to the above two models (21), thereby providing a
much sterner test of the ability of ASK1/p38 blockade to prevent diabetic glomerulosclerosis.
Second, we performed an intervention study and demonstrated that GS-444217 could inhibit p38
activation, halt glomerulosclerosis, reduce glomerular inflammation and improve renal function in
established diabetic nephropathy. Indeed, the results of the current study are an important
component of the preclinical data that support a current phase 2 trial evaluating an ASK1 inhibitor,
GS-4997, in patients with stage 3/4 diabetic kidney disease (27).

The ability of GS-444217 to reduce collagen deposition in the diabetic glomerulus is consistent
with previous studies in which p38 inhibitors have been shown to reduce renal fibrosis in non-
diabetic models of kidney disease (19; 28; 29). In addition, Ask1-/− mice show a profound
suppression of p38 activation and tissue fibrosis in models of both renal and cardiac disease (4; 30;
31).

A second mechanism whereby ASK1 inhibition reduced diabetic glomerular damage is through the
inhibition of macrophage infiltration and inhibition of mRNA levels of pro-inflammatory mediators.
Previous studies have shown that macrophages cause glomerular injury in experiment models of
diabetic nephropathy (32; 33). This is consistent with the reduction in CCL2 mRNA levels and
macrophage infiltration seen in the tubulointerstitium in the obstructed kidney in Ask1-/− mice (4).

The aggressive nature of disease development in diabetic Nos3-/− mice is attributed to the presence
of hypertension. However, the ASK1 inhibitor protected against diabetic glomerulosclerosis despite
having no effect on hypertension in Nos3-/− mice. This is consistent with previous studies on Ask1-
/- mice, which are protected from end organ damage in the setting of hypertension. For example, in
models of angiotensin II and mineralocorticoid-driven hypertension, \textit{Ask1-/-} mice had reduced cardiac fibrosis despite unaltered hypertension \cite{30, 31}. In the current study, ASK1 inhibition with GS-444217 appears to reduce renal injury and improve renal function by directly decreasing inflammation and fibrosis in the diabetic kidney.

The loss of podocytes and development of albuminuria in diabetic \textit{Nos3-/-} mice were unaffected by ASK1 inhibitor treatment in our early and late intervention studies. This suggests that activation of ASK1/p38 signalling is not involved in the mechanism of diabetic albuminuria. However, there are some limitations in this interpretation. Recent studies of \textit{Nos3-/-} mice has shown that they are highly susceptible to glucose induced podocyte damage and show marked albuminuria 2 weeks after STZ administration \cite{34}, which was the time at which we began ASK1 inhibitor treatment in the early intervention study. Therefore, it is possible that ASK1 inhibition may have been commenced too late to have an effect on the induction of podocyte damage/loss and albuminuria. Previous studies in both diabetic and non-diabetic kidney disease have shown that p38 inhibitors can reduce albuminuria \cite{9, 10, 28, 35}, arguing that ASK1/p38 signalling may contribute to albuminuria in some contexts.

Early intervention with the ASK1 inhibitor saw a reduction in HbA$_{1c}$ levels in diabetic \textit{Nos3-/-} mice despite no detectable difference in fasting blood glucose or plasma insulin levels. The reason for this discrepancy is unclear. However, no change in HbA$_{1c}$ levels or other parameters of diabetes was seen in the late intervention study, demonstrating that halting established diabetic glomerulosclerosis with GS-444217 was not due to modification of the diabetic state.

While there are several members of the MAP3K family that can induce p38 activation, we have shown that ASK1 plays a non-redundant and essential role in activating p38 in the diabetic kidney. This is consistent with a role for ASK1 in high glucose-induced p38 activation in cultured
glomerular mesangial cells (36), and the various stresses present in diabetes (e.g. oxidative stress, endoplasmic reticular stress, hyperglycaemia and angiotensin II) that are known to activate ASK1/p38 signalling (37). However, activation of p38 MAPK signalling can operate independent of ASK1 in kidney cells as shown by the unaltered LPS and IL-1 induced p38 activation and biological responses seen in Ask1-/− tubular epithelial cells (4). Thus, ASK1 is a context-dependent regulator of p38 activation, which is particularly important in settings of oxidative stress owing to the regulation of ASK1 activation by antioxidant proteins such as thioredoxin. One limitation of the present study is that currently available commercial antibodies are not sensitive enough to detect ASK1 in tissue sections or for detection of ASK1 phosphorylation in kidney tissues.

ASK1/p38 signaling has been implicated in other aspects of diabetes. ASK1/p38 signaling is involved in stress-induced death of pancreatic β cells and in the induction of endothelial cell senescence induced by high glucose (38; 39). Ask1-/− mice have reduced levels of maternal diabetes-induced endoplasmic reticulum stress in the developing embryo (40). Indirect blockade of ASK1 signaling can protect against diabetic cardiomyopathy (41; 42), although transgenic mice over-expressing a kinase dead form of ASK1 are not protected from diabetic neuropathy (43). Therefore, pathological signalling by ASK1 may be a common contributor to diabetic end organ damage in the pancreas, heart and kidney.

In conclusion, treatment with an ASK1 inhibitor halted the progression of glomerulosclerosis and renal inflammation and improved renal function in an aggressive model of diabetic nephropathy. This protective effect was attributed to blockade of ASK1-dependent p38 signaling. This study identifies ASK1 as a new therapeutic target in diabetic nephropathy and a related ASK1 inhibitor is now being tested in a phase 2 clinical trial (NCT02177786).
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**FIGURE LEGENDS**

**Figure 1. Effect of GS-444217 (ASK1i) on p38 MAPK activation in the diabetic kidney.** (A) Western blot analysis of phosphorylated (p)-p38 in whole kidney samples, and (B) quantification of p-p38 compared to the tubulin control, in the early intervention study. Groups of diabetic Nos3-/- mice were fed standard chow (no treatment, No Tx) or chow with 0.1% GS-444217 from week 2 after STZ injection until being killed on week 8. Age-matched non diabetic Nos3-/- mice were used as controls (No STZ). (C) Western blot analysis, and (D) quantification, of p-p38 in whole kidney samples, in the late intervention study. Groups of diabetic Nos3-/- mice were fed standard chow (No Tx) or chow with 0.1% GS-444217 from week 8 after STZ injection until being killed on week 15. Age-matched non diabetic Nos3-/- mice were used as controls (No STZ). ***P<0.001. (E-G) Kidney immunostaining for phospho-p38 (brown) at week 8 (E-G) and week 15 (H-J) of diabetes. Immunostaining for p-p38 was seen in glomeruli, tubules and the interstitium of non-diabetic Nos3-/- control kidneys at week 8 (E) and week 15 (H). An increase in p-p38 was evident in diabetic kidneys, particularly within glomeruli and dilated tubules at week 8 (F), and was similarly elevated at week 15 (I). GS-444217 treatment largely abrogated the p-p38 staining in diabetic kidneys at week 8 (G) and week 15 (J). Original magnification: (E-G) x250.

**Figure 2. Effect of GS-444217 (ASK1i) on the development of diabetes in Nos3-/- mice.** Groups of diabetic Nos3-/- mice were fed standard chow (no treatment, No Tx) or chow with 0.1% GS-444217 from week 2 after STZ injection until being killed on week 8. Age-matched non diabetic Nos3-/- mice were used as controls (No STZ). (A) Fasting body weight, (B) fasting blood glucose (FBG), (C) HbA1c levels, and (D) plasma insulin levels. *P<0.05, **P<0.01, ***P<0.001, NS not significant.
Figure 3. Effect of GS-444217 (ASK1i) on albuminuria, podocyte loss and renal function in early diabetes. Groups of diabetic Nos3-/− mice were fed standard chow (no treatment, No Tx) or chow with 0.1% GS-444217 from week 2 after STZ injection until being killed on week 8. Age-matched non diabetic Nos3-/− mice were used as controls (No STZ). (A) Urine albumin:creatinine ratio, (B) number of WT1+ podocytes per glomerular cross-section, and (C) renal function measured by serum cystatin-C levels. **P<0.05, ***P<0.001.

Fig. 4. Effect of GS-444217 (ASK1i) on glomerular damage in early diabetes. (A-C) PAS stained kidney sections. (A) Non-diabetic control Nos3-/− kidney. (B) Significant PAS-stained deposits were seen in glomeruli after 8 weeks of diabetes in mice with no treatment. (C) Treatment with GS-444217 over weeks 2 to 8 reduced glomerular PAS-stained deposits. (D-F) Immunostaining for collagen IV (brown). (D) Non-diabetic control Nos3-/− kidney showing staining of collagen IV in the glomerular basement membrane. (E) Significant mesangial deposition of collagen IV after 8 weeks of diabetes with no treatment. (F) Treatment with GS-444217 suppressed glomerular collagen IV deposition. (G-I) Immunostaining for CD68+ macrophages (brown) with weak hematoxylin and PAS staining. (G) Non-diabetic control Nos3-/− kidney showing a single CD68+ cell in a glomerulus. (H) Increased numbers of glomerular CD68+ cells were seen in an untreated mice after 8 weeks of diabetes. (I) Treatment with GS-444217 reduced the number of glomerular CD68+ cells in diabetic mice. (J) Image analysis of the area of glomerular collagen IV staining. (K) Quantification of the number of glomerular CD68+ macrophages. Original magnification (A-I) x400. *P<0.05, **P<0.01, ***P<0.001.

Figure 5. Effect of GS-444217 (ASK1i) on the progression of diabetes in Nos3-/− mice. Groups of diabetic Nos3-/− mice were fed standard chow (no treatment, No Tx) or chow with 0.1% GS-444217 from week 8 after STZ injection until being killed on week 15. Age-matched non diabetic
Nos3-/- mice were used as controls (No STZ). (A) Fasting body weight (FBW), (B) fasting blood glucose (FBG), and (C) HbA1c levels.

**Figure 6. Effect of GS-444217 (ASK1i) on albuminuria, podocyte loss and renal function in progressive diabetes.** Groups of diabetic Nos3-/- mice were fed standard chow (no treatment, No Tx) or chow with 0.1% GS-444217 from week 8 after STZ injection until being killed on week 15. Age-matched non diabetic Nos3-/- mice were used as controls (No STZ). (A) Urine albumin:creatinine ratio, (B) number of WT1+ podocytes per glomerular cross-section, and (C) renal function measured by serum cystatin-C levels. ***P<0.001 vs STZ-No Tx.

**Figure 7. Effect of GS-444217 (ASK1i) on kidney fibrosis in progressive diabetes.** (A-D) Immunostaining for collagen IV. (A) Non-diabetic control Nos3-/- kidney. (B) Significant mesangial deposition of collagen IV after 8 weeks of diabetes with no treatment. (C) Enhanced glomerular collagen IV deposition after 15 weeks of diabetes with no treatment. (D) GS-444217 treatment over weeks 8 to 15 prevents further glomerular collagen IV deposition. (E) Image analysis of the area of glomerular collagen IV staining. (F-J) Real time PCR analysis of whole kidney mRNA levels for: (F) collagen IV, (G) collagen I, (H) fibronectin (FN), (I) TGF-β1, and (J) PAI-1. Original magnification (A-D) x400. *P<0.05, **P<0.01, ***P<0.001.

**Figure 8. Effect of GS-444217 (ASK1i) on kidney inflammation and tubular damage in progressive diabetes.** (A-B) Immunostaining for CD68+ macrophages (brown) with weak hematoxylin and PAS staining. (A) A Nos3-/- kidney at week 15 of diabetes showing accumulation of glomerular CD68+ macrophages. (B) Treatment with GS-444217 reduced glomerular CD68+ macrophages in diabetic mice. (C) Quantification of the number of glomerular CD68+ macrophages. (D-G) Real-time PCR analysis of whole kidney mRNA levels for: (D) CD68, (E) CCL2, (F) TNF-α, and (G) KIM-1. *P<0.05, **P<0.01, ***P<0.001.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

A. Urine ACR (g/mol) over weeks after STZ treatment.

B. WT1+ cells/gcs comparison among groups:
- No STZ
- STZ-No Tx
- STZ-ASK1i
- Non-Diabetic

C. Serum Cystatin-C (ng/ml) over weeks after STZ treatment.
Figure 7
Figure 8
### Supplementary Table 1: qPCR probe and primer sequences

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<td>Collagen IV</td>
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<td>KIM-1</td>
<td>CAACAGACCACCAAC</td>
<td>TTAAACCAGAGATTCCACA</td>
<td>TGGAGGAGTGGAGGAGTAGAGA</td>
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