View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

© 2009 International Society of Nephrology

see commentary on page 815

Transforming growth factor-β2 upregulates sphingosine kinase-1 activity, which in turn attenuates the fibrotic response to TGF-β2 by impeding CTGF expression

Shuyu Ren[1], Andrea Babelova[2], Kristin Moreth[2], Cuiyan Xin[1], Wolfgang Eberhardt[2], Anke Doller[2], Hermann Pavenstädt[3], Liliana Schaefer[2], Josef Pfeilschifter[2] and Andrea Huwiler[1]

[1]Institute of Pharmacology, University of Bern, Bern, Switzerland; [2]Pharmazentrum Frankfurt, Klinikum der J.W.Goethe-Universität, Frankfurt/Main, Germany and [3]Medizinische Klinik und Poliklinik D, Wilhelm-Universität Münster, Münster, Germany

Transforming growth factor-β2 (TGF-β2) stimulates the expression of pro-fibrotic connective tissue growth factor (CTGF) during the course of renal disease. Because sphingosine kinase-1 (SK-1) activity is also upregulated by TGF-β, we studied its effect on CTGF expression and on the development of renal fibrosis. When TGF-β2 was added to an immortalized human podocyte cell line we found that it activated the promoter of SK-1, resulting in upregulation of its mRNA and protein expression. Further, depletion of SK-1 by small interfering RNA or its pharmacological inhibition led to accelerated CTGF expression in the podocytes. Overexpression of SK-1 reduced CTGF induction, an effect mediated by intracellular sphingosine-1-phosphate. *In vivo*, SK-1 expression was also increased in the podocytes of kidney sections of patients with diabetic nephropathy when compared to normal sections of kidney obtained from patients with renal cancer. Similarly, in a mouse model of streptozotocin-induced diabetic nephropathy, SK-1 and CTGF were upregulated in podocytes. In SK-1 deficient mice, exacerbation of disease was detected by increased albuminuria and CTGF expression when compared to wild-type mice. Thus, SK-1 activity has a protective role in the fibrotic process and its deletion or inhibition aggravates fibrotic disease.

Kidney International (2009) **76,** 857–867; doi:10.1038/ki.2009.297; published online 5 August 2009

KEYWORDS: CTGF; diabetic nephropathy; podocytes; sphingosine kinase-1; streptozotocin; TGFβ

Correspondence: *Andrea Huwiler, Institute of Pharmacology, University of Bern, Friedbühlstrasse 49, CH-3010 Bern, Switzerland. E-mail: huwiler@pki.unibe.ch*

Received 29 April 2008; revised 10 June 2009; accepted 23 June 2009; published online 5 August 2009

Glomerular visceral epithelial cells, also denoted as podocytes, build regularly spaced, interdigitated foot processes that form the filtration slit and are connected by a thin membrane-like slit diaphragm that has an important role in the regulation of glomerular ultrafiltration.[1,2] Damage of podocytes, which can be triggered by various factors, including oxidative and inflammatory stress, regularly leads to foot process effacement and results in a destruction of the filtration barrier and uncontrolled loss of proteins into the urinary space, which is a hallmark of many forms of kidney diseases.[1–4] It was suggested that proteinuria is a result of sublytic complement C5b-9 attack on podocytes, which leads to the induction of various genes involved in the production of oxidants, proteases, and growth factors, including the transforming growth factor-β (TGFβ) and the connective tissue growth factor (CTGF).[5]

TGFβ has an important role in many cell types.[6,7] It initiates cellular signaling by binding to the TGFβ-receptor type II, which interacts with the TGFβ receptor type I (TGFβRI) and results in the activation of the kinase activity of TGFβRI. In turn, Smad proteins are phosphorylated and translocate to the nucleus to act as transcription factors.[6,7] In the kidney, TGFβ has a key effect in the induction of renal fibrosis.[8–10] Not surprisingly, in this context, TGFβ is the best known stimulator of CTGF, which is considered to have a crucial role in wound repair and fibrosis,[11,12] and is also significantly upregulated in the course of fibrotic renal disease.[13] CTGF is proposed to stimulate fibroblast proliferation, matrix production, and promote adhesion and migration of many cell types, although the detailed molecular mechanisms remain to be deciphered.[14–16]

Sphingolipids are important structural components of cell membrane, which may exert additional functions as signaling molecules under various physiological and pathophysiological conditions.[17,18] Sphingosine-1-phosphate (S1P) represents one of these bioactive molecules generated from sphingosine by the action of sphingosine kinases (SKs). S1P is involved in the regulation of pleiotropic cell responses,

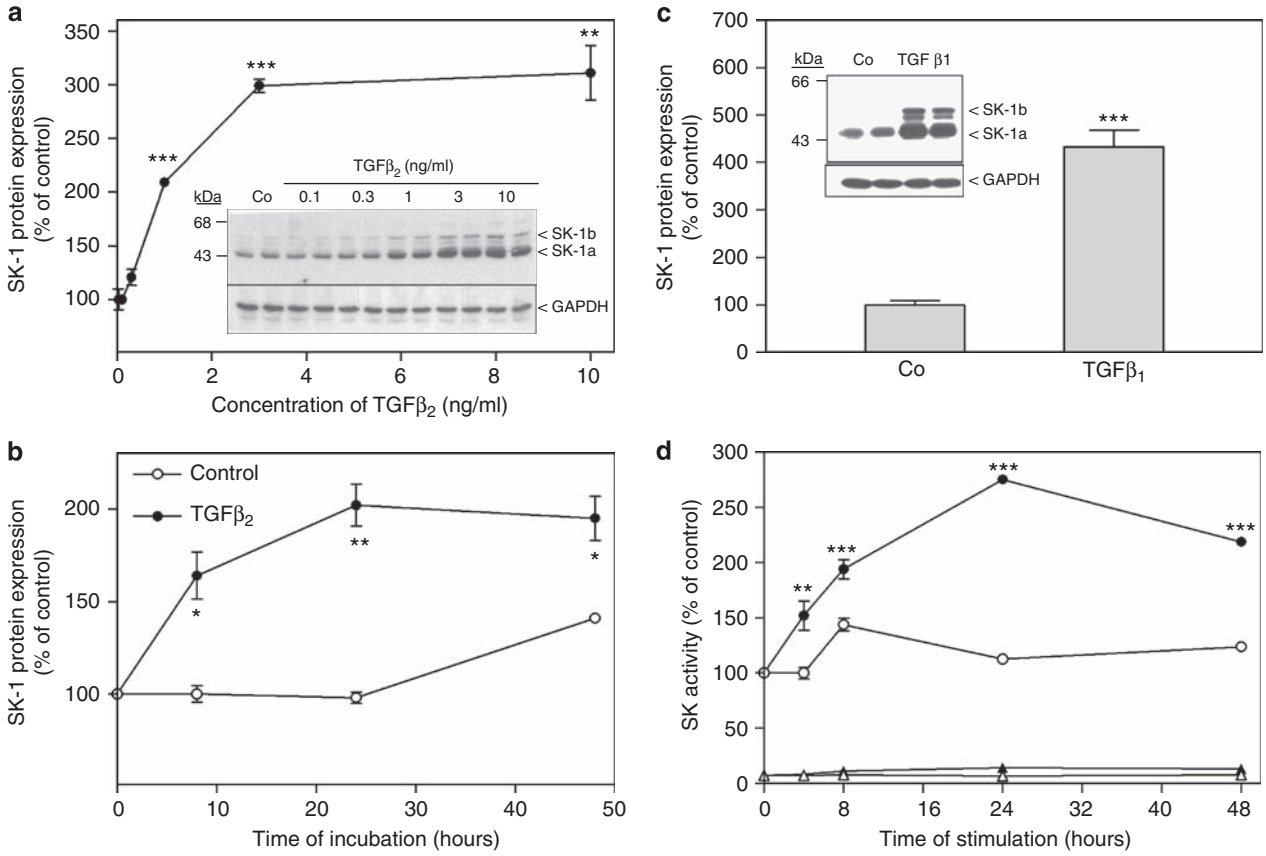

Figure 1 | Effect of TGFβ2 on sphingosine kinase-1 protein expression and activity in human podocytes. Podocytes were stimulated for 24 h with the indicated concentrations of $TGF\beta_2$ (**a**); or for the indicated time periods with either vehicle (**b**, open symbols) or 5 ng/ml of $TGF\beta_2$, (**b**, closed symbols); or for 24 h with 5 ng/ml of $TGF\beta_1$ (**c**). Thereafter, cell lysates were processed for western blot analysis (**a**–**c**) using specific antibodies against human SK-1 (**a** and **c**, insets, upper panels) at a dilution of 1:1000, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (**a** and **c**, insets, lower panels) at a dilution of 1:2000. Bands corresponding to SK-1a were densitometrically evaluated and are expressed as percentage of control and means ± s.d. ($n = 3$) (**d**). Cells were stimulated for the indicated time periods with either vehicle (open symbols) or 5 ng/ml of $TGF\beta_2$ (closed symbols). Thereafter, cell lysates were taken for *in vitro* SK-1 (circles) or SK-2 (triangles) activity assays as described in the Materials and Methods. SK-1 activity in control samples was at 10,509 c.p.m./min/mg of protein; SK-2 activity in control samples was at 695 c.p.m./min/mg of protein. Data are expressed as percentage of unstimulated controls of SK-1 activity and means ± s.d. ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ were considered statistically significant when compared with vehicle-stimulated values.

such as cell proliferation and differentiation, survival, and migration.[17–19]

Two subtypes of SK have been identified, SK-1 and SK-2, which are both ubiquitously expressed[20,21] but show differential enzymatic characteristics in that SK-2 possesses a broader substrate specificity than does SK-1.[21] Total SK activity is stimulated in many cell types by various growth factors, and also by proinflammatory cytokines. Whereas the mechanism of SK-1 activation includes phosphorylation reactions, membrane translocation,[22,23] and transcriptional events,[24–26] the mechanism of SK-2 activation is still unclear.

In this study, we show that, in human podocytes, $TGF\beta_2$ not only induces the expression of profibrotic CTGF but also upregulates SK-1 activity. Depletion or inhibition of SK-1 leads to a higher CTGF expression, whereas overexpression of SK-1 causes a suppression of CTGF expression. This regulation is also observed in a mouse model of streptozotocin (STZ)-induced diabetic nephropathy, in which SK-1 and CTGF were both upregulated in podocytes under disease conditions. Strikingly, in SK-1-deficient mice, an even higher expression of CTGF was detected when compared with wild-type mice and this correlated with a deterioration of the disease.

RESULTS

$TGF\beta_2$ upregulates SK-1 activity by stimulating its gene transcription

Stimulation of human podocytes with $TGF\beta_2$ led to a concentration (Figure 1a) and time-dependent (Figure 1b) increase of SK-1 protein expression, as detected by western blot analysis. The effect persisted for at least 48 h (Figure 1b). A similar effect on SK-1 protein expression was also observed when using $TGF\beta_1$ (Figure 1c). The increased SK-1 protein expression correlated with an increased SK-1 activity (Figure 1d). To discriminate the activity of SK-1 from SK-2, the composition of the *in vitro* kinase buffer was changed. In buffer conditions, including 1 M KCl, in which SK-1 activity is inhibited by more than 90% but SK-2 is still active, only a minor activity of SK-2 was observed (Figure 1d, triangles)

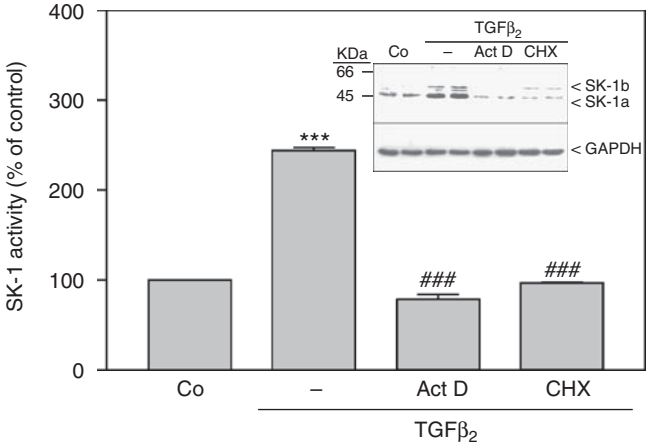

Figure 2 | Effects of cycloheximide and actinomycin D on TGFβ$_2$-induced SK-1 protein expression and activity in human podocytes. Podocytes were pretreated for 30 min using vehicle (–), cycloheximide (CHX; 10 μg/ml), or actinomycin D (Act D; 5 μg/ml) before stimulation for 24 h with vehicle (Co) or TGFβ$_2$ (5 ng/ml). Thereafter, cell lysates were processed for western blot analysis of SK-1 (inset, upper panel) or GAPDH (inset, lower panel), or were taken for an *in vitro* SK-1 activity assay as described in the Materials and Methods. Data in the inset are representative of three experiments. Results in the graph are expressed as % of control values and means ± s.d. ($n = 3$). ***$P < 0.001$ was considered statistically significant when compared with vehicle-stimulated control values; ###$P < 0.001$ compared with TGFβ-stimulated values.

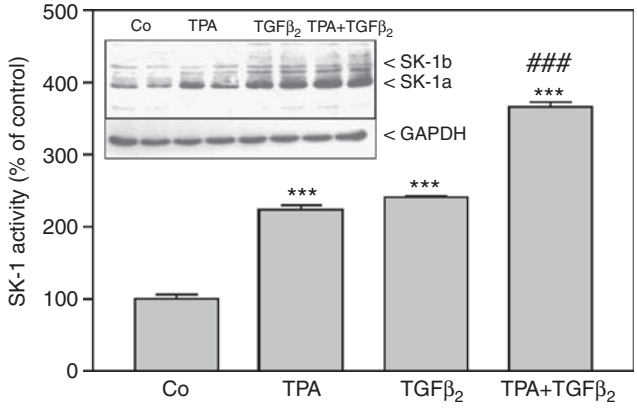

Figure 3 | Effect of phorbol ester on TGFβ-induced SK-1 protein expression and activity in human podocytes. Confluent podocytes were treated for 24 h with either vehicle (Co), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (100 nM), TGFβ$_2$ (5 ng/ml), or TPA plus TGFβ$_2$ as indicated. Thereafter, cell lysates were processed for western blot analysis of SK-1 (inset, upper panel) or GAPDH (inset, lower panel), or taken for an *in vitro* SK-1 activity assay as described in the Materials and Methods. Data in the inset are representative of three experiments. Results in the graph are expressed as percentage of control values and means ± s.d. ($n = 3$). ***$P < 0.001$ was considered statistically significant when compared with vehicle-stimulated values; ###$P < 0.001$ compared with TGFβ-stimulated control values.

compared with SK-1 activity. This remaining SK-2 activity was not significantly altered by TGFβ treatment (Figure 1d).

The upregulation of SK-1 protein expression was due to increased *de-novo* protein synthesis and gene transcription, as the protein synthesis inhibitor, cycloheximide, and the transcriptional inhibitor, actinomycin D, blocked TGFβ-triggered SK-1 activity (Figure 2) and SK-1 protein expression (Figure 2, inset). Previously, we had shown that phorbol ester is also a potent inducer of SK-1 expression and activity in human endothelial cells[26] and mesangial cells.[27] Moreover, in podocytes, phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-acetate) upregulated SK-1 activity (Figure 3) and expression (Figure 3, inset). Co-treatment of cells with TPA plus TGFβ led to an additive effect on SK-1 activity and expression (Figure 3), suggesting that at least two independent signaling pathways are involved in a maximal expressional upregulation of SK-1.

Moreover, TGFβ$_2$ also upregulated SK-1, but not SK-2, mRNA expression levels, as shown by quantitative PCR analysis (Figure 4a). To determine whether the increased SK-1 mRNA levels occurred by an increased promoter activation,[26,28] SK-1 promoter studies were performed by using a luciferase-containing vector fused with a 2492 bp fragment of the human SK-1a promoter. Transfection of podocytes with this construct, followed by TGFβ$_2$ stimulation, revealed a more than twofold enhancement of SK-1 promoter activity (Figure 4b, right panel). Sequence analysis of the SK-1 promoter revealed a putative Smad-3 (−1237 bp to –1229 bp) and a putative Smad-4-binding site (−1581 bp to –1572 bp) (Figure 4b left panel). Both sites were mutated by site-directed mutagenesis. However, only the Smad-4-binding site turned out to be functional, as a mutation of this site, but not of the Smad-3-binding site, resulted in a loss of TGFβ-triggered promoter activation (Figure 4b, right panel).

To elucidate the mechanism by which TGFβ$_2$ induces SK-1 expression, various inhibitors of protein kinase modules were tested. The induction of SK-1 by TGFβ$_2$ required TGFβ$_2$ receptor type I, as in the presence of a TGF-β receptor type I kinase inhibitor, the TGFβ$_2$-induced SK-1 protein expression was completely abolished (Figure 5a). In contrast, neither the classical MAPK/ERK cascade inhibitor U0126, the general protein kinase C inhibitor Ro 318220, inhibitors of p38-MAPK SB 203580, SB 202190, and PD 169316, nor the JNK inhibitor SP 600125 attenuated SK-1 expression (Figure 5a). Furthermore, depletion by small interfering RNA (siRNA) of the co-regulatory Smad-4, a downstream member of the TGFβ signaling device, completely abrogated TGFβ-triggered SK-1 upregulation (Figure 5b).

Suppressive role of SK-1 on TGFβ-induced CTGF expression

To determine whether the upregulation of SK-1 by TGFβ$_2$ has functional consequences for podocytes, a well-established TGFβ-regulated target gene was investigated, that is, the CTGF, which is considered an important factor in fibrotic cell response.[13,15,16]

The protein expression and subsequent secretion of CTGF, which was increased by TGFβ (Figure 6a, open columns), were negatively regulated by SK-1, as the depletion of SK-1 by

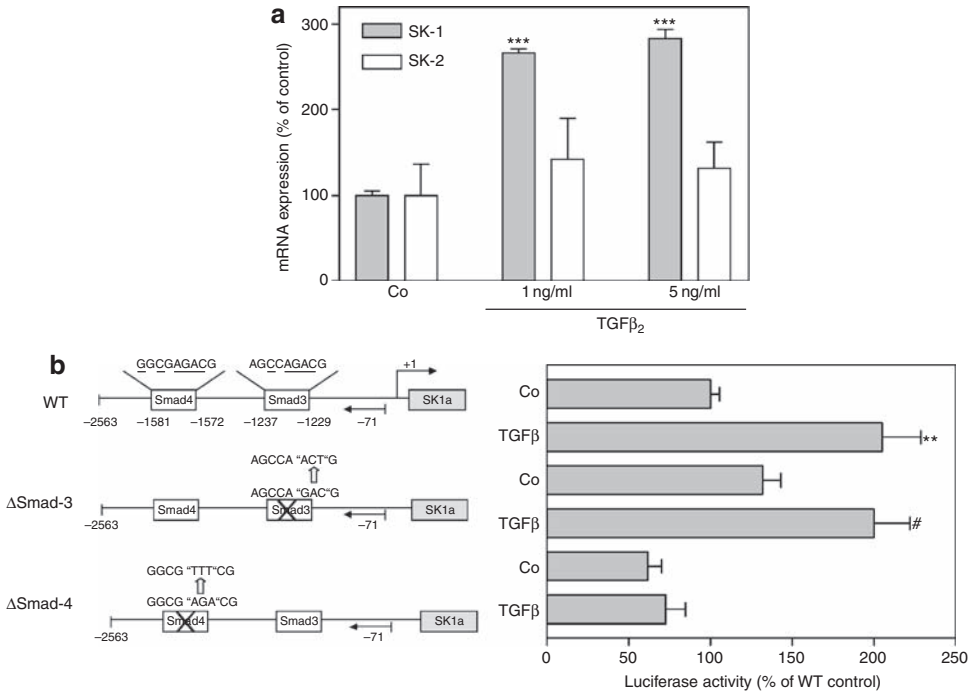

Figure 4 | Effect of TGFβ_2 on SK-1 mRNA expression and promoter activity in human podocytes. (**a**) Podocytes were stimulated for 4 h with either vehicle (Co) or the indicated concentrations of TGFβ_2. Thereafter, RNA was extracted and used for quantitative PCR of SK-1 (closed columns) or SK-2 (open columns), as described in the Materials and Methods. Results are expressed as percentage of control values and means ± s.d. ($n = 3$). (**b**) The 2492 bp wild-type (WT) promoter fragment containing the putative Smad-3 and Smad-4-binding sites, and the generated mutants are shown (left panel). Podocytes were transfected with DNA containing the WT 2492 bp fragment, the Smad-3-mutated fragment (ΔSmad-3), or the Smad-4-mutated fragment (ΔSmad-4), as described in the Materials and Methods. Cells were stimulated for 24 h with either vehicle (Co) or 5 ng/ml of TGFβ_2 (right panel). The ratio between firefly and Renilla luciferase activities was calculated. Results are expressed as percentage of WT control and means ± s.d. ($n = 3$); $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$ were considered statistically significant when compared with the corresponding control values.

siRNA led to an amplified expression and secretion of CTGF protein under basal as well as under TGFβ-stimulated conditions (Figure 6a, closed columns). The transfection of podocytes with siRNA completely reduced the high expression of SK-1 obtained by TGFβ stimulation when compared with scrambled siRNA transfection (Figure 6b).

To exclude the possibility that the effects observed by siRNA transfection are due to unrelated off-target phenomena, we additionally tested a recently developed specific SK-1 enzyme inhibitor, SKI II.[29] As seen in Figure 7, CTGF protein expression induced by TGFβ was also enhanced in the presence of increasing concentrations of the inhibitor. Moreover, a stable overexpression of SK-1 in podocytes by a lentiviral transduction method resulted in a massive accumulation of SK-1a and SK-1b in the transduced cells (Figure 8, upper panel). In these cells, the secreted CTGF was hardly detectable (Figure 8, lower panel), which further stresses the negative regulation of CTGF by SK-1.

In line with the negative effect of SK-1 on CTGF expression, its product S1P should reduce CTGF expression. To this end, cells were stimulated with exogenous S1P, which acted through surface S1P-receptors. However, this regimen rather stimulated CTGF expression, which was already observed at early time points of 2 h and 4 h (Figure 9a), consistent with previous reports in other cell types.[30,31] After 24 h, CTGF expression was comparable between control- and S1P-stimulated cells (Figure 9b). As an intracellular way of action of S1P is also possible and has been proposed by various groups, we tested the effect of caged S1P.[32] This S1P derivative does not bind to S1P-receptors but penetrates the cells and, on illumination, the protective group is cleaved off to generate 'active' S1P in the cytoplasm.[32] Figure 9b shows that increasing concentrations of intracellularly released S1P indeed reduced CTGF expression.

SK-1 and CTGF are overexpressed in podocytes from diabetic nephropathy patients

To determine whether SK-1 is expressed *in vivo* in human podocytes, renal sections of healthy controls and of patients with diabetic nephropathy, according to Mogensen,[33,34] were examined by immunohistochemical staining. SK-1 was hardly stained in podocytes of healthy samples, but in patients with diabetic nephropathy, staining was much more pronounced (Figure 10, arrowhead). In addition, an increased CTGF expression was detected in diabetic podocytes (Figure 10, arrow), confirming our previous study in which we showed a coexpression of CTGF and TGF-β in podocytes of diabetic patients.[35] Thus, it is conceivable that

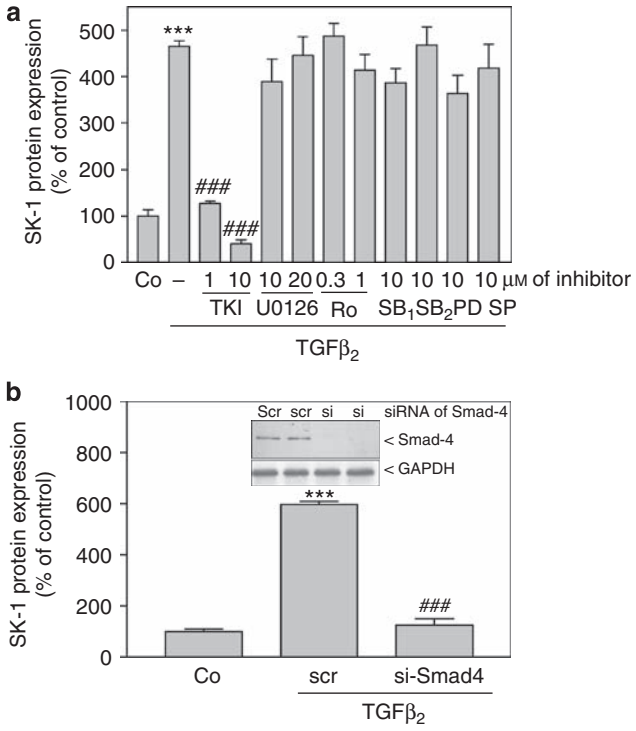

Figure 5 | Effect of various kinase inhibitors or of Smad-4 depletion on SK-1 protein expression in human podocytes. (**a**) Podocytes were pretreated for 30 min using the indicated concentrations (in μM) of TGFβRI inhibitor (TKI), U0126, Ro 318220 (Ro), SB 203580 (SB_1), SB 202190 (SB_2), PD 169316 (PD), or SP600125 (SP) before stimulation with either vehicle (Co,–) or 5 ng/ml of $TGF\beta_2$. (**b**) Podocytes were transfected with vehicle (Co), small interfering RNA (siRNA) for human Smad-4 (si), or a scrambled siRNA (scr) as recommended by the manufacturer. At 48 h after transfection, cells were stimulated with either vehicle or 5 ng/ml of $TGF\beta_2$ for 24 h. Thereafter, cells were processed for western blot analysis of SK-1 (1:1000), Smad-4 (1:1000) (**b**, inset upper panel), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000) (**b**, inset, lower panel). Bands corresponding to SK-1a were densitometrically evaluated and are expressed as percentage of control and means ± s.d. ($n = 3$). ***$P < 0.001$ was considered statistically significant when compared with vehicle-stimulated values; ###$P < 0.001$ compared with TGFβ-stimulated control values.

the crosstalk between TGF-β and SK-1 signaling regulates the CTGF expression in podocytes, emphasizing the relevance of our *in vitro* findings for the development of renal disease.

SK-1-deficient mice develop a more severe STZ-induced nephropathy

In a further approach to address the function of SK-1 in regulating CTGF expression *in vivo*, STZ-mediated type 1 diabetes was induced in (C57BL/6) *SK-1*$^{+/+}$ and *SK-1*$^{-/-}$ mice. Diabetic mice of both genotypes developed hyperglycemia with increased kidney-to-body weight ratios at day 30 compared with nondiabetic mice, with no distinguishable changes among the diabetic groups, irrespective of the *SK-1* gene dose (Table 1). The only genotype-specific difference observed as a sign of progressive nephropathy was a sustained increase in albuminuria ($P < 0.05$) at days 20 and 30 in diabetic *SK-1*$^{-/-}$ compared with *SK-1*$^{+/+}$ diabetic mice (Figure 11a). In agreement with our findings in human diabetic glomeruli, STZ-induced diabetes mellitus resulted in an enhanced protein expression of SK-1 in glomeruli, most likely located in podocytes and mesangial cells (Figure 11b, immunostaining, 30 days), and in tubules (data not shown) from *SK-1*$^{+/+}$ diabetic kidneys. This increment was further confirmed by western blot for SK-1 in nondiabetic and diabetic *SK-1*$^{+/+}$ kidneys (Figure 11c and d). The specificity of SK-1-detection in mouse kidney homogenates by western blotting was confirmed by a preincubation of anti-SK-1 antibody with recombinant mouse SK-1 (Figure 11c, 'negative control'). Furthermore, by performing *in situ* hybridization, we detected an increased glomerular (Figure 11e) and tubular (data not shown) expression of CTGF mRNA in diabetic kidneys from mice of both genotypes. However, *SK-1* deficiency markedly enhanced CTGF mRNA expression in podocytes, as shown by *in situ* hybridization (Figure 11e) and its semi-quantification (Figure 11f).

DISCUSSION

In this study, we have shown that *in vitro* $TGF\beta_2$ potently upregulates SK-1 expression and activity in glomerular podocytes. *In vivo*, SK-1 is highly upregulated in podocytes of kidneys from diabetic nephropathy patients, as well as in kidneys from mice with STZ-induced nephropathy. We show for the first time that, mechanistically, TGFβ upregulated SK-1 expression by stimulating the SK-1 promoter activity (Figure 4b). Promoter sequence analyses revealed two putative Smad-binding elements, one Smad-3 and one Smad-4-binding site (see Figure 4a). By site-directed mutagenesis of these two sites, we identified the Smad-4-binding site as a functional site responsible for the TGFβ-triggered effect. Previously, it was shown that Sp1 and AP-2 sites were involved in phorbol ester-induced SK-1 promoter activation in the leukemia cell line, MEG-O1.[36] In addition, we recently identified a functional hypoxia-responsive element that mediates hypoxia-triggered SK-1 upregulation in human endothelial cells,[37] and a STAT5-binding site that mediates prolactin-triggered SK-1 upregulation in the human breast cancer cell line, MCF7.[28] All these data underline that SK-1 activity is tightly regulated by transcriptional mechanisms. Obviously, the TGFβ/Smad signaling cascade represents one possible mode of upregulation of SK-1 expression and activity, whereas phorbol ester-activated PKC signaling is a further separate mode of SK-1 expression and activation. In combination, TGFβ plus phorbol ester exerted an additive effect on SK-1, as one expects from independent signaling pathways acting on the same cell response. Together, this suggests that for a full persistent activation of SK-1, activation of both the PKC and Smad signaling modules is required. A similar upregulation of SK-1 mRNA and activity by TGFβ was also reported for dermal fibroblasts, although the mechanism was not further addressed.[38]

In functional terms, our data show that the activation of SK-1 by TGFβ is involved in the regulation of the important

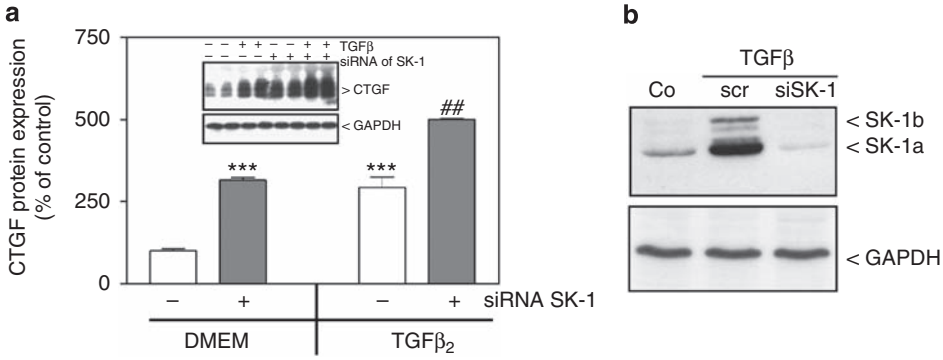

Figure 6 | Effect of SK-1 depletion by small interfering RNA (siRNA) on TGFβ-induced connective tissue growth factor (CTGF) protein expression in human podocytes. (**a**) Podocytes were transfected with either a scrambled siRNA (−) or siRNA for SK-1 (+) before stimulation for 24 h with either vehicle (DMEM) or TGFβ_2 (5 ng/ml). Thereafter, supernatants were taken for protein precipitation using trichloroacetic acid and processed for western blot analysis of secreted CTGF (inset, upper panel). The corresponding cell lysates were subjected to a western blot analysis using an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (inset, lower panel). (**b**) Protein lysates of unstimulated untransfected cells (Co), TGFβ-stimulated scrambled transfected (scr) or TGFβ-stimulated SK-1-siRNA transfected (siSK1) cells were analyzed by western blots for protein expression of SK-1 (upper panel) or GAPDH (lower panel). Bands corresponding to CTGF were densitometrically evaluated and are depicted as bars. Results are expressed as percentage of control values and means ± s.d. ($n = 4$). ***$P < 0.001$ was considered statistically significant when compared with scrambled transfected unstimulated values; ##$P < 0.01$ compared with SK-1 siRNA-transfected unstimulated values.

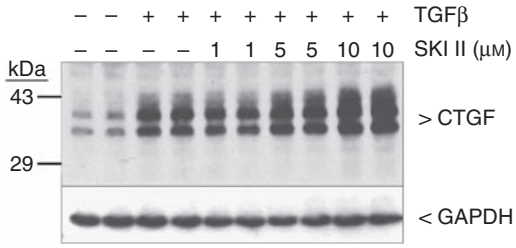

Figure 7 | Effect of SK-1 inhibition on TGFβ-stimulated connective tissue growth factor (CTGF) protein expression in human podocytes. Podocytes were pretreated for 30 min with either vehicle (−) or the indicated concentrations of the SK-1 inhibitor (SKI II; in μM) before stimulation with TGFβ_2 (5 ng/ml; +) for 24 h. Thereafter, supernatants were taken for protein precipitation using trichloroacetic acid and for the determination of secreted CTGF protein (upper panel) by western blot analysis using an antibody against CTGF at a dilution of 1:1000. The corresponding cell lysates were taken for GAPDH analysis (lower panel). Data are representative of four experiments giving similar results.

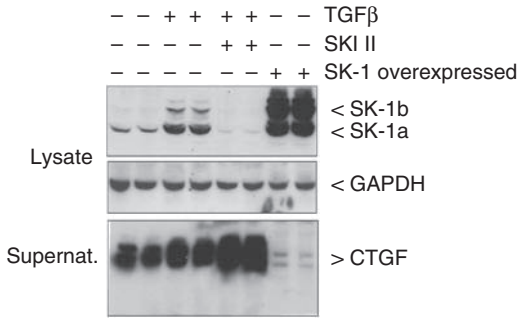

Figure 8 | Effect of SK-1 overexpression on connective tissue growth factor (CTGF) protein expression in human podocytes. Podocytes were taken either untransduced and stimulated for 24 h with TGFβ_2 (5 ng/ml) in the absence or presence of SKI II (10 μM) or transduced with a lentiviral construct containing SK-1a + b. Thereafter, cell lysates were taken for determination of SK-1 (upper panel) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (middle panel) expressions, and supernatants (supernat.) were taken for protein precipitation using trichloroacetic acid and for the determination of secreted CTGF protein (lower panel) by western blot analysis using antibodies against SK-1 (dilution 1:2000, upper panel), GAPDH (dilution 1:3000, middle panel), and CTGF (dilution 1:1000, lower panel). Data are representative of three experiments giving similar results.

profibrotic factor, CTGF. CTGF is upregulated in most fibrotic diseases, including human crescentic glomerulonephritis[39] and experimental proliferative glomerulonephritis.[40] In view of these data, SK-1 may act as a brake in a fibrotic event by impeding CTGF expression. This is further supported by our *in vivo* model showing that SK-1-deficient mice not only express higher amounts of CTGF but also develop a more severe diabetic nephropathy when treated with STZ and compared with SK-1 wild-type mice.

Most notably, we found that the suppressive effect of SK-1 is exclusively mediated by locally generated intracellular S1P, and not by extracellular S1P acting through S1P-receptors. In fact, extracellular S1P even has an opposite effect and can increase CTGF expression and may thereby even worsen a fibrotic process. The mechanisms by which intracellular S1P suppresses CTGF protein expression remain unknown, because the identity of intracellular S1P targets is still not defined. Various studies have shown that intracellular S1P triggers an intracellular Ca^{2+} mobilization, which occurs independently of a phospholipase C/IP_3-driven Ca^{2+} release.[41] Thus, it is tempting to speculate that Ca^{2+} channels are directly affected by intracellular S1P.

Inhibition of SK-1 has been proposed as a useful approach to treat tumor growth, as shown in a mouse xenograft model.[29,42] Additional *in vivo* effects of these SK-1 inhibitors have not yet been addressed. Our data show that SK-1 inhibition also results in an increased CTGF expression in

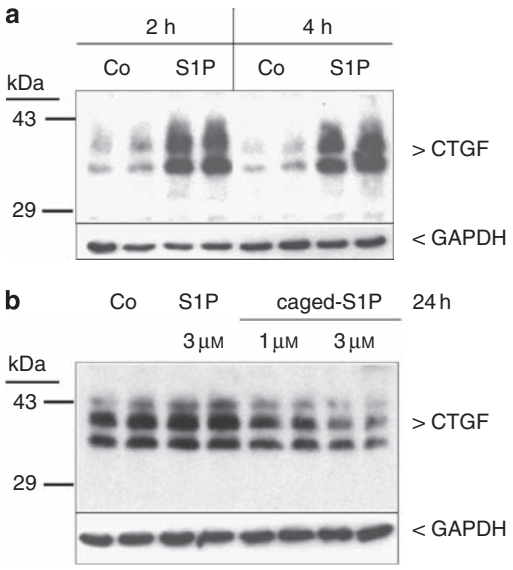

Figure 9 | Effects of exogenously applied S1P and intracellularly generated S1P on connective tissue growth factor (CTGF) expression. (**a**) Podocytes were stimulated for 2 h and 4 h with either vehicle (Co) or 3 μM of exogenous S1P. (**b**) Cells were stimulated for 24 h with either vehicle (Co), 3 μM of exogenous S1P, or 3 μM and 10 μM of caged S1P (illuminated for 30 s at 360 nM and with a change of medium before stimulation). Thereafter, supernatants were taken for protein precipitation using trichloroacetic acid and for the determination of secreted CTGF protein (upper panels) by western blot analysis using an antibody against CTGF at a dilution of 1:1000. The corresponding cell lysates were taken for GAPDH analysis (lower panels). Data are representative of four independent experiments giving similar results.

kidney cells and this could lead to adverse side effects when using SK-1 inhibitors *in vivo*. However, it should be noted that the relation of CTGF with tumor development and growth is still controversial. On the one side, a reduced CTGF expression was reported for various tumors, including lung cancer,[43] leading to speculations that CTGF could be a tumor suppressor. Consistent with this idea, increased CTGF levels were shown to inhibit the metastatic activity of human lung cancer cells.[44,45] Most recently, it was shown that CTGF levels were decreased in Wilms tumor (nephroblastoma),[46] and that recombinant CTGF inhibited the proliferation of a Wilms tumor cell line. On the other side, CTGF was proposed to promote angiogenesis[47] and tumorigenesis of prostate cancer cells.[48] Moreover, blocking CTGF action by a recently developed neutralizing antibody, FG-3019, inhibited pancreatic tumor growth and metastasis.[49,50] Overall, these data raise the question whether inhibition of SK-1 could have a beneficial or deteriorating effect on renal fibrotic diseases. In this context, it has previously been reported that STZ-induced diabetic nephropathy in rats correlated with an accumulation of S1P in glomeruli isolated from kidney cortex.[51] Moreover, high glucose conditions led to an increased SK-1 activity in endothelial cells[52] and in vascular smooth muscle cells,[53] proposing a role for SK-1 in diabetic vasculo- and nephropathies. However, these studies did not address whether SK-1 positively promotes diabetic nephropathy or whether SK-1 is induced as a rescue mechanism to counter-regulate disease progression. Recently, Kono *et al.*[54] reported that SK-1 is upregulated in fibrotic foci in a mouse

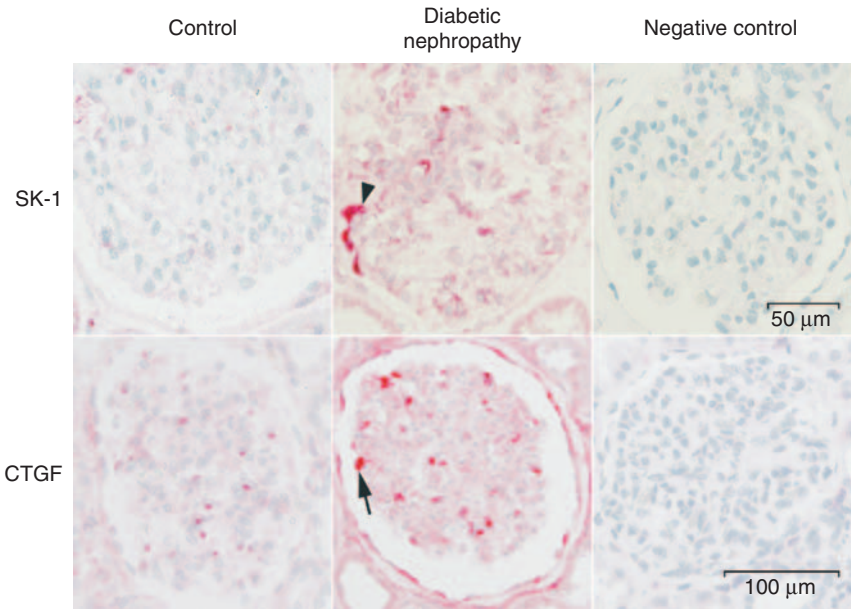

Figure 10 | Immunohistochemical staining of SK-1 in kidney sections from patients with diabetic nephropathy. Glomerular SK-1 and connective tissue growth factor (CTGF) expression in normal human kidney (control, left panels) and in kidneys from patients with manifest diabetic nephropathy (middle panels) were detected by immunohistochemical staining using an anti-SK-1 antibody as described in the Materials and Methods. The arrowhead points to SK-1 expression and the arrow points to CTGF expression in podocytes. Renal sections from patients with diabetic nephropathy were used as negative controls by omitting the primary antibody (right panels). Bars indicate magnification.

Table 1 | Effects of streptozotocin (STZ)-induced diabetes (day 30) on body weight, kidney weight, and blood glucose levels in *SK-1*$^{+/+}$ and *SK-1*$^{-/-}$ mice

	Nondiabetic (*n*=6)		Diabetic (*n*=6)	
	SK-1$^{+/+}$	*SK-1*$^{-/-}$	*SK-1*$^{+/+}$	*SK-1*$^{-/-}$
Body weight, day 0 (g)	27.3 ± 0.6	26.2 ± 1.7	26.9 ± 1.3	25.9 ± 1.8
Body weight, day 30 (g)	28.1 ± 0.1	27.4 ± 1.5	23.3 ± 2.1[a]	22.5 ± 1.7[b]
Kidney weight (mg)	205 ± 21	199 ± 23	227 ± 18[a]	261 ± 10[b]
(Kidney weight/body weight) × 10^3	7.3 ± 0.9	7.3 ± 0.9	9.9 ± 1.2[a]	11.7 ± 0.7[b]
Blood glucose (mg per 100 ml)	96 ± 5	95 ± 4	422 ± 32[a]	426 ± 36[b]

Data are given as means ± s.d., $P<0.05$.
[a]For diabetic *SK-1*$^{+/+}$ versus nondiabetic *SK-1*$^{+/+}$ mice.
[b]For diabetic *SK-1*$^{-/-}$ versus nondiabetic *SK-1*$^{-/-}$ mice.

model of pulmonary fibrosis, although no SK-1$^{-/-}$ mice were used in that study to prove the *in vivo* contribution of SK-1 to the disease. Our studies using SK-1$^{-/-}$ mice now suggest that SK-1 induced under disease conditions exerts a protective function and its depletion results in a more severe kidney disease.

In summary, our data have shown that in human podocytes, TGFβ potently upregulates SK-1 expression and activity, which critically contributes to a negative regulation of CTGF expression, which exerts a key effect in organ fibrosis. Furthermore, the *in vivo* model of STZ-induced diabetic nephropathy showed that SK-1 exerted a protective function, as SK-1$^{-/-}$ mice developed a more severe disease that correlated with an enhanced CTGF expression. However, this study leaves open tantalizing questions with regard to extracellular and intracellular S1P and whether inhibition of SK-1 *in vivo* is a good or bad effect. This thrilling question definitely needs to be addressed in future studies.

MATERIALS AND METHODS

Chemicals

TGF-β was obtained from R&D Systems (Wiesbaden, Germany); cycloheximide, actinomycin D, and STZ were purchased from Sigma-Aldrich Fine Chemicals (Deisenhofen, Germany); and all inhibitors were obtained from Merck Biosciences (Schwalbach, Germany). The CTGF (L-20) antibody, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (V-18) antibody, and the human Smad-4 siRNA (sc-29484) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany); the SK-1-specific antibody was generated and characterized as previously described.[25,26]

Cell culture

A human immortalized podocyte cell line was cultured as described.[55] Cells were normally cultured at 33 °C. For experiments, cells were cultured at 37 °C for a further 10 days. These cells were characterized by the expression of the podocyte-specific protein, nephrin.[1,55] For synchronization, cells were incubated for 16 h in Dulbecco's modified Eagle's medium, including 0.1 mg/ml of fatty acid-free bovine serum albumin.

siRNA transfections

Gene silencing of human SK-1 was performed exactly as previously described.[26,28] Silencing efficiency was confirmed by western blot analysis.

Quantitative PCR analysis

Real-time PCR was performed using a BioRad iQ-iCycler Detection System (BioRad, München, Germany). Primer sequences were as follows: human GAPDH (accession number: NM_002046): forward: GCTCTCTGCTCCTCCTGTTC; reverse: CGCCCAATACGACCAA ATCC; human SK-1 (accession number: NM_021972): forward: GGGCTTCATTGCTGATGTGGAC; reverse: TGCCTGCCATTACAA CTGTCC; IQ5 Optical System Software (version 2.0, BioRad) was used to analyze real-time and end point fluorescence.

Western blot analysis

Stimulated cells were homogenized in lysis buffer and processed as previously described.[25,26] Protein of 30 μg was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and subjected to western blot analysis. To prove that the anti-human SK-1 antibody also specifically recognized mouse SK-1 in mouse kidney homogenates, the antibody was preincubated with recombinant mouse SK-1a (SPHK1a, Biomol, Hamburg, Germany) for 12 h at 4 °C before an overnight incubation at 4 °C with the western blot of nondiabetic and diabetic homogenates of SK-1$^{+/+}$ kidneys.

SK activity assay

In vitro kinase reactions were performed exactly as previously described.[26] For SK-1 activity, 0.5% Triton X100 was included to block SK-2 activity.[21] For SK-2 activity, 1 M KCl was included to block SK-1 activity.[21]

SK-1 promoter studies

A 2492 bp fragment of the human SK-1a promoter was cloned by reverse transcriptase PCR using the following primers: forward with an *Nhe*1 side: GCTAGCAGGTGCAGGACCCATCATTC; reverse with a *Hind*III side: AAGCTTCCTGCCTTCAGCTCCTTATC. The promoter fragments were fused into the pGL3 luciferase reporter gene-containing vector (Promega, Mannheim, Germany). Cell transfections and reporter assays were performed exactly as described.[26] Values for relative SK-1 promoter activities were calculated from the ratio of firefly/Renilla luciferase activities.

Promoter mutations

All mutated constructs were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using the following mutation primers: for pGL-hSK1a-ΔSmad3: forward: GCCGCCTTCTAGCCAACTGCCTAGGACGAGCGGC (GAC to ACT); for pGL-hSK1a-ΔSmad4: forward: TGGGCGGCGGGCGTTT

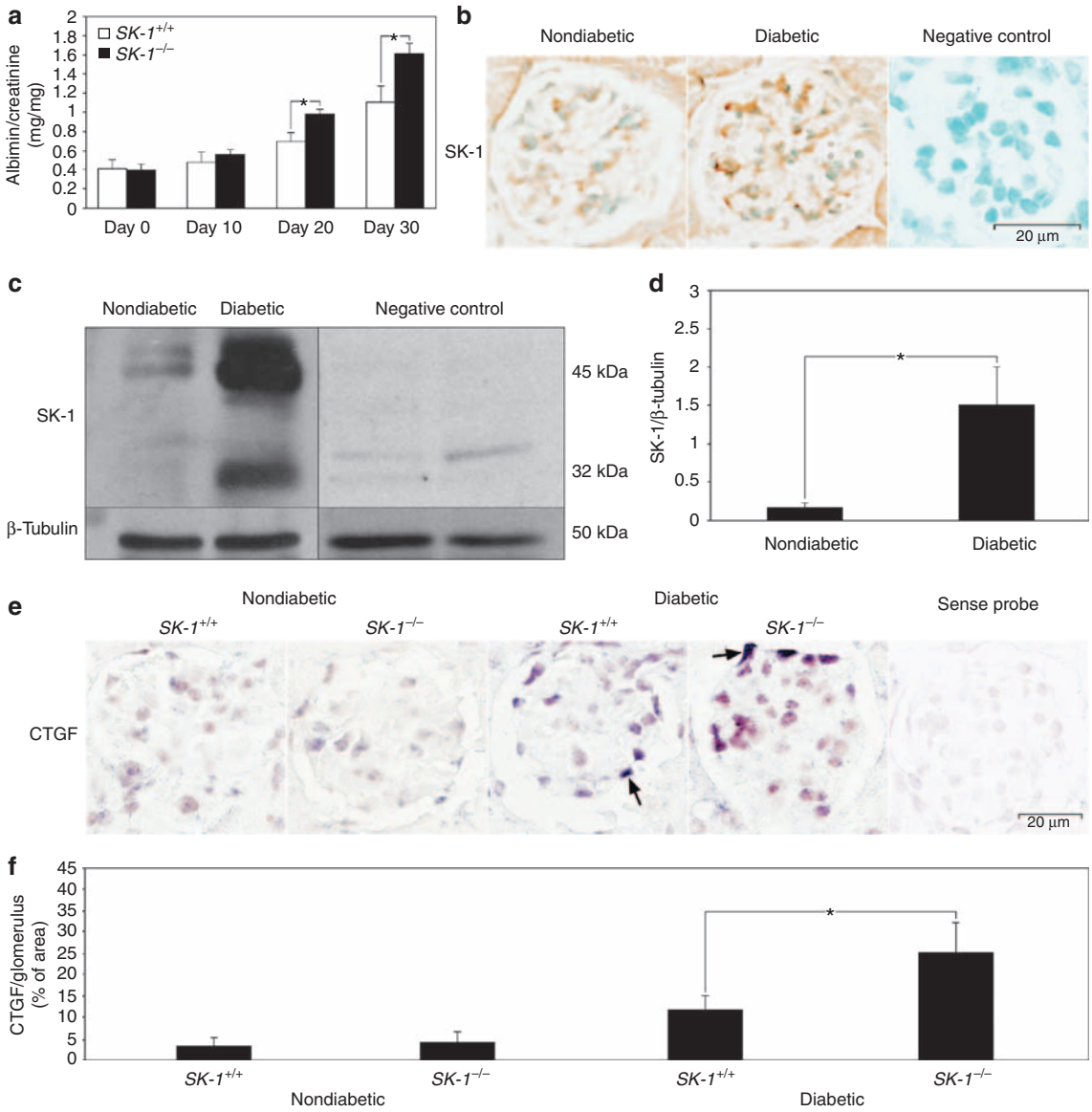

Figure 11 | Effect of SK-1 deficiency on albuminuria and connective tissue growth factor (CTGF) expression in streptozotocin-induced diabetes mellitus in mice. (**a**) Urinary albumin excretion normalized to creatinine levels in the urine from nondiabetic and diabetic *SK-1*$^{+/+}$ and *SK-1*$^{-/-}$ mice before induction of diabetes mellitus (day 0), as well as 10, 20, and 30 days after the last streptozotocin (STZ) injection. The asterisk between bars indicates statistical differences between diabetic *SK-1*$^{+/+}$ and *SK-1*$^{-/-}$ mice, $P<0.05$, $n=6$. (**b**) Immunostaining (brown color) for SK-1 in glomeruli of nondiabetic and diabetic *SK-1*$^{+/+}$ kidneys after 30 days of STZ diabetes. Renal sections from diabetic *SK-1*$^{+/+}$ mice were used as negative controls by omitting the primary antibody. Bars indicate magnification. (**c**) Western blot and (**d**) quantification of SK-1 (45 kDa plus 32 kDa) and β-tubulin in homogenates of diabetic kidneys from *SK-1*$^{+/+}$ mice at day 30 of diabetes. Anti-SK-1 antibody preincubated with recombinant mouse SK-1 was used for western blotting as a negative control, indicating specificity of antibody detection (**c**). The asterisk between bars indicates statistical differences between diabetic and nondiabetic *SK-1*$^{+/+}$ kidneys, $P<0.05$, $n=3$ (**d**). Data are given as means ± s.d. of the ratios of optical density for SK-1 (45 kDa plus 32 kDa) to β-tubulin. (**e**) *In situ* hybridization of CTGF in glomeruli of nondiabetic and diabetic *SK-1*$^{+/+}$ and *SK-1*$^{-/-}$ kidneys after 30 days of STZ diabetes and (**f**) its semi-quantification expressed as the ratio (%) of the glomerular CTGF mRNA-positive area to the whole glomerular area from 10 randomly selected high-power fields. Arrows indicate expression of CTGF in podocytes. Bars indicate magnification. The asterisk between bars indicates statistical differences between diabetic and nondiabetic *SK-1*$^{+/+}$ kidneys, $^{*}P<0.05$, $n=5$.

CGCCCCGGCCTGGAGA (GAG to TTT). The mutated sites were verified by sequencing.

Tissues for histological investigations

For controls, tumor-free kidney samples were obtained from six patients undergoing nephrectomy for renal carcinoma. Diabetic changes were examined in tissues from two kidney biopsies with no evidence of malignancy and in six tumor-free samples from carcinoma kidneys from patients with manifest diabetic nephropathy. The clinical data of these patients have been described previously.[35,56] The study was approved by the Ethical Committee of the Medical Faculty, University of Muenster.[35]

Tissue samples were fixed and prepared as previously described.[56] Paraffin sections (2–6 μm) were stained using periodic acid–Schiff reagent to ascertain the absence of tumor infiltration and were processed for immunohistochemistry using a rabbit anti-human SK-1 antibody at a dilution of 1:500 and a secondary alkaline phosphatase-coupled anti-rabbit antibody. Counterstaining was performed using Mayer's hemalaun. For mouse tissue, serial sections (3–5 μm) of paraffin-embedded mouse kidney samples were used. Counterstaining was performed using methyl green. The specificity of staining was ascertained using negative controls by omitting the primary antibody and by using non-immune serum 'unspecific' IgG.

In situ hybridization in mouse kidney

A DNA probe comprising nucleotides 1–492 of mouse CTGF cDNA was used for *in situ* hybridization of formaldehyde-fixed and RNAse-free paraffin-embedded kidney sections as described previously.[56] To semi-quantify the expression of CTGF, 10 randomly selected nonoverlapping fields of the renal cortex were examined under high-power-field magnification and the ratio (%) of the glomerular CTGF-mRNA-positive area to the whole glomerular area was determined using the Soft Imaging System (Olympus, Tokyo, Japan). Mean values of at least five kidneys per group were averaged.

Animal experiments

All animal experiments were conducted in accordance with the German Animal Protection Law and were approved by the Ethics Review Committee of the District Governments of Muenster and Darmstadt, Germany. To induce type-1 diabetes, 2-month-old male *SK-1*$^{+/+}$(C57BL/6) and *SK-1*$^{-/-}$ mice[57] were intraperitoneally injected with STZ (45 mg/kg of body weight) dissolved in 100 mM sodium citrate buffer (pH 4.5) for four consecutive days. Control animals were injected with citrate buffer. Mice that had developed glycosuria received a subcutaneous insulin implant (Linplant^{-}) (Linshin, Ontario, Canada) to prevent ketoacidosis. Blood glucose levels were controlled every 10 days (Haemo-Glukotest; Roche Diagnostics, Mannheim, Germany). Urinary glucose and ketones were measured using reagent strips (Keto-Diastix Reagent Strips, Bayer Vital, Leverkusen, Germany). Urinary creatinine excretion was determined with the Creatinine Assay Kit (NatuTec, Frankfurt/Main, Germany), and the Albuwell M ELISA Kit (Exocell, Philadelphia, PA, USA) was used for quantification of urinary albumin excretion. Kidneys ($n = 6$ per group) were harvested and analyzed 30 days after induction of diabetes.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance, followed by Bonferroni's *post hoc* test for multiple comparisons.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by the Swiss National Science Foundation (3100A0-111806), the German Research Foundation (FOG784, GRK757/2, PF361/6–1, HU842/4–1, EXC147, SFB 815, SCHA1082/2–1), the EC (FP6: LSHM-CT-2004–005033), the Wilhelm-Sander Foundation, the IZKF (Muenster), and the ECCPS.

REFERENCES

1. Pavenstädt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* 2003; **83**: 253–307.
2. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J Clin Invest* 2001; **108**: 1583–1587.
3. Tryggvason K, Wartiovaara J. Molecular basis of glomerular permselectivity. *Curr Opin Nephrol Hypertens* 2001; **10**: 543–549.
4. Asanuma K, Mundel P. The role of podocytes in glomerular pathobiology. *Clin Exp Nephrol* 2003; **7**: 255–259.
5. Couser WG, Nangaku M. Cellular and molecular biology of membranous nephropathy. *J Nephrol* 2006; **19**: 699–705.
6. Massague J, Gomis RR. The logic of TGFβ signaling. *FEBS Lett* 2006; **580**: 2811–2820.
7. Massague J. TGF-beta signal transduction. *Annu Rev Biochem* 1998; **67**: 753–791.
8. Border WA, Noble NA. Cytokines in kidney disease: the role of transforming growth factor-beta. *Am J Kidney Dis* 1993; **22**: 105–113.
9. Tamaki K, Okuda S, Ando T *et al.* TGF-beta 1 in glomerulosclerosis and interstitial fibrosis of adriamycin nephropathy. *Kidney Int* 1994; **45**: 525–536.
10. Yu L, Border WA, Huang Y *et al.* TGF-beta isoforms in renal fibrogenesis. *Kidney Int* 2003; **64**: 844–856.
11. Leask A, Abraham DJ. The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology. *Biochem Cell Biol* 2003; **81**: 355–363.
12. Leask A, Abraham DJ. TGF-β signaling and the fibrotic response. *FASEB J* 2004; **18**: 816–827.
13. Gupta S, Clarkson MR, Duggan J *et al.* Connective tissue growth factor: potential role in glomerulosclerosis and tubulointerstitial fibrosis. *Kidney Int* 2000; **58**: 1389–1399.
14. Perbal B. CCN proteins: multifunctional signaling regulators. *Lancet* 2004; **363**: 62–64.
15. Shimo T, Nakanishi T, Nishida T *et al.* Connective tissue growth factor induces proliferation, migration, and tube formation of vascular endothelial cells *in vitro*, and angiogenesis *in vivo*. *J Biochem* 1999; **126**: 137–145.
16. Crean JK, Furlong F, Finlay D *et al.* Connective tissue growth factor [CTGF]/CCN2 stimulates mesangial cell migration through integrated dissolution of focal adhesion complexes and activation of cell polarization. *FASEB J* 2004; **18**: 1541–1543.
17. Huwiler A, Kolter T, Pfeilschifter J *et al.* Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim Biophys Acta* 2000; **1485**: 63–99.
18. Spiegel S, Milstien S. Sphingosine 1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 2003; **4**: 397–407.
19. Hla T. Signaling and biological actions of sphingosine 1-phosphate. *Pharmacol Res* 2003; **47**: 401–407.
20. Melendez AJ, Carlos-Dias E, Gosink M *et al.* Human sphingosine kinase: molecular cloning, functional characterization and tissue distribution. *Gene* 2000; **251**: 19–26.
21. Liu H, Sugiura M, Nava VE *et al.* Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J Biol Chem* 2000; **275**: 19513–19520.
22. Pitson SM, Moretti PA, Zebol JR *et al.* Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. *EMBO J* 2003; **22**: 5491–5500.
23. Pitson SM, Xia P, Leclerq TM *et al.* Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. *J Exp Med* 2005; **201**: 49–54.
24. Chen XL, Grey JY, Thomas S *et al.* Sphingosine kinase-1 mediates TNF-alpha-induced MCP-1 gene expression in endothelial cells: upregulation by oscillatory flow. *Am J Physiol Heart Circ Physiol* 2004; **287**: H1452–H1458.
25. Döll F, Pfeilschifter J, Huwiler A. The epidermal growth factor stimulates sphingosine kinase-1 expression and activity in the human mammary carcinoma cell line MCF7. *Biochim Biophys Acta* 2005; **1738**: 72–81.
26. Huwiler A, Döll F, Ren S *et al.* Histamine increases sphingosine kinase-1 expression and activity in the human arterial endothelial cell line EA. hy 926 by a PKC-α-dependent mechanism. *Biochim Biophys Acta* 2006; **1761**: 367–376.
27. Klawitter S, Hofmann LP, Pfeilschifter J *et al.* Extracellular nucleotides induce migration of renal mesangial cells by upregulating sphingosine kinase-1 expression and activity. *Br J Pharmacol* 2007; **150**: 271–280.
28. Döll F, Pfeilschifter J, Huwiler A. Prolactin upregulates sphingosine kinase-1 expression and activity in the human breast cancer cell line MCF7 and triggers enhanced proliferation and migration. *Endocr Relat Cancer* 2007; **14**: 325–335.
29. French KJ, Schrecengost RS, Lee BD *et al.* Discovery and evaluation of inhibitors of human sphingosine kinase. *Cancer Res* 2003; **63**: 5962–5969.

30. Xin C, Ren S, Kleuser B *et al.* Sphingosine 1-phosphate cross-activates the Smad signaling cascade and mimics transforming growth factor-beta-induced cell responses. *J Biol Chem* 2004; **279**: 35255–35262.
31. Wuehlich S, Schneider N, Hinkmann F *et al.* Induction of connective tissue growth factor (CTGF) in human endothelial cells by lysophosphatidic acid, sphingosine-1-phosphate, and platelets. *Atherosclerosis* 2004; **175**: 261–268.
32. Meyer zu Heringdorf D, Liliom K, Schaefer M *et al.* Photolysis of intracellular caged sphingosine-1-phosphate causes Ca2+ mobilization independently of G-protein-coupled receptors. *FEBS Lett* 2003; **554**: 443–449.
33. Mogensen CE, Christensen CK, Vittinghus E. The stages in diabetic renal disease with emphasis on the stage of incipient diabetic nephropathy. *Diabetes* 1983; **32**(Suppl. 2): 64–78.
34. Mogensen CE. Microalbuminuria as a predictor of clinical diabetic nephropathy. *Kidney Int* 1987; **31**: 673–689.
35. Wahab NA, Schaefer L, Weston BS *et al.* Glomerular expression of TSP-1, TGF-β and CTGF at different stages of diabetic nephropathy and their interdependent roles in mesangial response to diabetic stimuli. *Diabetologia* 2005; **48**: 2650–2660.
36. Nakade Y, Banno Y, T-Koizumi K *et al.* Regulation of sphingosine kinase 1 gene expression by protein kinase C in a human leukemia cell line, MEG-O1. *Biochim Biophys Acta* 2003; **1635**: 104–116.
37. Schwalm S, Döll F, Römer I *et al.* Sphingosine kinase-1 is a hypoxia-regulated gene that stimulates migration of human endothelial cells. *Biochem Biophys Res Commun* 2008; **368**: 1020–1025.
38. Yamanaka M, Shegogue D, Pei H *et al.* Sphingosine kinase 1 (SPHK1) is induced by transforming growth factor-beta and mediates TIMP-1 up-regulation. *J Biol Chem* 2004; **279**: 53994–54001.
39. Kanemoto K, Usui J, Nitta K *et al.* *In situ* expression of connective tissue growth factor in human crescentic glomerulonephritis. *Virchows Arch* 2004; **444**: 257–263.
40. Ito Y, Goldschmeding R, Bende RJ *et al.* Kinetics of connective tissue growth factor expression during experimental proliferative glomerulonephritis. *J Am Soc Nephrol* 2002; **12**: 472–484.
41. Meyer zu Heringdorf D. Lysophospholipid receptor-dependent and -independent calcium signaling. *J Cell Biochem* 2004; **92**: 937–948.
42. French KJ, Upson JJ, Keller SN *et al.* Antitumor activity of sphingosine kinase inhibitors. *J Pharmacol Exp Ther* 2006; **318**: 596–603.
43. Chien W, Yin D, Gui D *et al.* Suppression of cell proliferation and signaling transduction by connective tissue growth factor in non-small cell lung cancer cells. *Mol Cancer Res* 2006; **4**: 591–598.
44. Chang CC, Shih JY, Jeng YM *et al.* Connective tissue growth factor and its role in lung adenocarcinoma invasion and metastasis. *J Natl Cancer Inst* 2004; **96**: 364–375.
45. Chang CC, Lin MT, Lin BR *et al.* Effect of connective tissue growth factor on hypoxia-inducible factor 1alpha degradation and tumor angiogenesis. *J Natl Cancer Inst* 2006; **98**: 984–995.
46. Li MH, Sanchez T, Pappalardo A *et al.* Induction of antiproliferative connective tissue growth factor expression in Wilms' tumor cells by sphingosine-1-phosphate receptor 2. *Mol Cancer Res* 2008; **6**: 1649–1656.
47. Brigstock DR. Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* 2002; **5**: 153–165.
48. Yang F, Tuxhorn JA, Ressler SJ *et al.* Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. *Cancer Res* 2005; **65**: 8887–8895.
49. Dornhofer N, Spong S, Bennewith K *et al.* Connective tissue growth factor-specific monoclonal antibody therapy inhibits pancreatic tumor growth and metastasis. *Cancer Res* 2006; **66**: 5816–5827.
50. Aikawa T, Gunn J, Spong SM *et al.* Connective tissue growth factor-specific antibody attenuates tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of pancreatic cancer. *Mol Cancer Ther* 2006; **5**: 1108–1116.
51. Geoffroy K, Troncy L, Wiernsperger N *et al.* Glomerular proliferation during early stages of diabetic nephropathy is associated with local increase of sphingosine-1-phosphate levels. *FEBS Lett* 2005; **579**: 1249–1254.
52. Wang L, Xing XP, Holmes A *et al.* Activation of the sphingosine kinase-signaling pathway by high glucose mediates the proinflammatory phenotype of endothelial cells. *Circ Res* 2005; **97**: 891–899.
53. You B, Ren A, Yan G *et al.* Activation of sphingosine kinase-1 mediates inhibition of vascular smooth muscle cell apoptosis by hyperglycemia. *Diabetes* 2007; **56**: 1445–1453.
54. Kono Y, Nishiuma T, Nishimura Y *et al.* Sphingosine kinase 1 regulates differentiation of human and mouse lung fibroblasts mediated by TGF-β1. *Am J Respir Cell Mol Biol* 2007; **37**: 395–404.
55. Greiber S, Münzel T, Kästner S *et al.* NAD(P)H oxidase activity in cultured human podocytes: effects of adenosine triphosphate. *Kidney Int* 1998; **53**: 654–663.
56. Schaefer L, Raslik I, Gröne HJ *et al.* Small proteoglycans in human diabetic nephropathy: discrepancy between glomerular expression and protein accumulation of decorin, biglycan, lumican and fibromodulin. *FASEB J* 2001; **15**: 559–561.
57. Hofmann LP, Ren S, Schwalm S *et al.* Sphingosine kinase 1 and 2 regulate the capacity of mesangial cells to resist apoptotic stimuli in an opposing manner. *Biol Chem* 2008; **389**: 1399–1407.