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Mazagova, Magdalena

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in Diabetic Kidney Injury

Magdaléna Mazagová

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Chapter 1

Introduction and the aims of the thesis

Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by high blood sugar (glucose) levels that result from defects in insulin secretion or action, or both. Normally, blood glucose level is tightly controlled by insulin, a hormone produced by the endocrine pancreas. When the blood glucose level rises, insulin is released from the pancreas to normalize the glucose level. There are several types of diabetes, of which type 1 and type 2 diabetes are the most common. Type 1 diabetes (T1DM, insulin-dependent diabetes or juvenile-onset diabetes) is characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas leading to insulin deficiency. Type 2 diabetes (T2DM, non-insulin-dependent diabetes or adult-onset diabetes) results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency.

Diabetes effects an estimated 6% of the adult population in Western society and over 2% worldwide[1]. T2DM accounts for more than 90% of all diabetics[2] and its worldwide prevalence is increasing and expected to grow by 3% per year due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. Worldwide, the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030, with the largest relative increase to occur in the Middle Eastern Crescent, sub-Saharan Africa and India[3].

The worldwide prevalence of T1DM is much lower and remains the predominant type of diabetes during childhood in many countries with relatively high incidence in Scandinavia[4]. Nevertheless, the incidence of T1DM is also increasing and the number of T1DM patients younger that 15 years in Europe is predicted to rise from 94.000 in 2005 to 160.000 in 2020[5].

Pharmacological treatment of T1DM focuses mainly on the administration of recombinant human insulin to compensate for the deficiency of insulin. In T2DM, several classes of drugs are used. Sulfonylurea derivatives such as glibenclamide act by increasing insulin release from the beta cells in the pancreas. Biguanides such as metformin act through reducing gluconeogenesis in the liver and enhancing cellular glucose uptake, both resulting in lower blood glucose levels. Glitazones act through several pathways, including a decrease in insulin resistance[6].

Proper pharmacological treatment and improved lifestyle can greatly reduce the severity of the chronic complications of diabetes. Nevertheless, diabetic complications such as nephropathy, cardiomyopathy, neuropathy and retinopathy remain common in patients. These complications are believed to result from hyperglycemic damage to small vessels, referred to as microvascular disease. Diabetes is also an important factor in accelerating atherosclerosis, thus leading to stroke, coronary heart disease, and microvascular diseases[7].

Diabetic nephropathy

Diabetic nephropathy is a common cause of chronic kidney failure (20-30%), resulting in an increased number of patients requiring end-stage renal failure management, particularly dialysis[8]. Diabetic nephropathy is characterized by specific renal morphological and functional alterations. Features of early diabetic renal changes are glomerular hyperfiltration, glomerular and renal hypertrophy, increased urinary albumin excretion, increased basement membrane thickness and mesangial cells expansion with accumulation of extracellular matrix (ECM) proteins. Advanced diabetic nephropathy is characterized by albuminuria, a decline in renal function, decreased creatinine clearance, glomerulosclerosis and interstitial fibrosis[9;10].

Renal fibrosis in diabetic nephropathy develops, in part, through activation of renal fibroblasts leading to secretion and remodeling of the ECM. Although activated resident renal fibroblasts (myofibroblast) are critical effectors of renal fibrosis some studies suggest that fibroblasts originating from epithelial–mesenchymal transition (EMT) may play an important additional role. Regardless of their origin, the development of renal fibrosis is dependent on the secretion of profibrotic mediators such as cytokines, protease inhibitors, growth factors and inflammatory mediators[11]. These include transforming growth factor β (TGF-β), connective tissue growth factor, angiotensin II, endothelin-1 and fibroblast growth factor[10;12;13].

Transforming growth factor family (TGF-β)

TGF-β is one of the most studied cytokines involved in diabetic nephropathy and is a member of a large family of structurally related cytokines (Figure 1). The family consists of more than 30 growth factors, including bone morphogenetic protein (BMP), inhibins and activins[14]. Members of the TGF-β family regulate a wide variety of biological processes, such as proliferation[15], differentiation, cytokine production and immune cell modulation[16].

A key role for TGF-β1 in the pathogenesis of renal fibrosis has been established in both experimental and clinical kidney disease. Intravascular injection of TGF-β into rats or

rabbits rapidly causes glomerulosclerosis[17]. Overexpression of TGF-β in normal, uninjured glomeruli by gene transfection produces glomerulosclerosis within days[18]. Moreover, transgenic mice with the TGF-β gene under control of the albumin promoter die of renal failure caused by a lesion that resembles rapidly progressive glomerulonephritis with tubulointerstitial fibrosis[19].

Also in diabetes, the functional role of TGF-β1 in renal fibrosis was demonstrated by studies in which the inhibition of TGF-β1 using neutralizing antibodies, decorin or antisense oligonucleotides, result in the prevention or amelioration of renal fibrosis[20-22].

Although, TGF-β appears to be the most prominent cytokine in renal fibrosis, other TGF family members may play an important modulatory role. An example of this is Bone Morphogenetic Protein-7 (BMP-7), which is expressed in the healthy kidney. Loss of renal BMP-7 expression is associated with the early development of diabetic nephropathy[23], possibly because the decrease of renal BMP-7 expression favors TGF-β driven renal fibrogenesis. In accord, treatment of streptozotocin induced diabetic mice with exogenous human recombinant BMP-7 effectively prevented and reversed renal fibrosis[24;25]. The interaction of TGF-β and BMP-7 has been studied at the molecular level and *in vitro* experiments demonstrate that the interaction of downstream signaling molecules are responsible for the antagonistic effects of BMP-7 on TGF-β mediated renal fibrogenesis[26].

TGF-β superfamily member signaling pathways

Members of the TGF-β superfamily signal through TGF-β type I and II receptors (Figure 2). Binding of the ligand to the type II receptor causes recruitment of the type I receptor into a heterodimeric receptor complex. Activation by phosphorylation of the type I receptor serine/threonine kinase induces serine phosphorylation of receptor Smads. In most tissues, TGF-β causes Smad2/3 activation, whereas BMP activates Smad1, 5, and 8. Subsequently, phosphorylated receptor Smads bind to the common Smad4 to form the Smad complex, which translocates into the nucleus to regulate target gene transcription. Two inhibitory Smads (Smad6 and Smad7) are amongst the target genes, providing the possibility of negative feedback control. It should be pointed out that within the TGF-β superfamily, both pathways cross-regulate each other to maintain homeostasis during normal physiology. This interaction can occur at different levels, including competition for receptors and Smads[27].

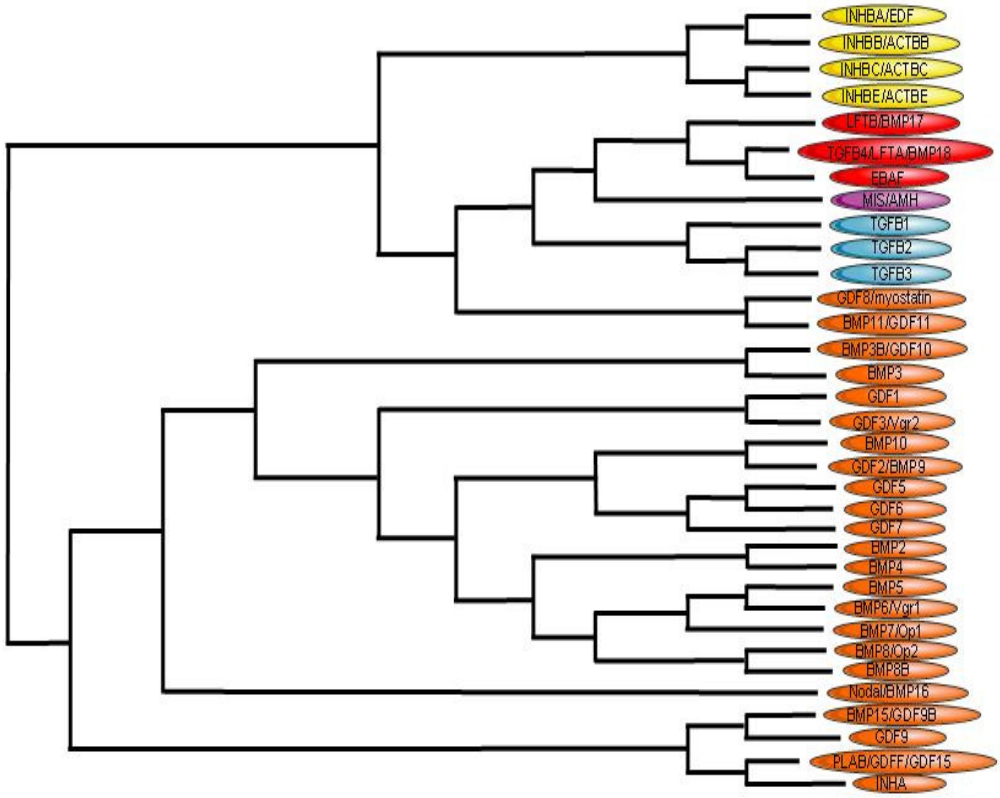

Figure 1: The TGF-ß superfamily. Dendrogram of all human TGF-ß superfamily members (colour differentiation based on subgroups).

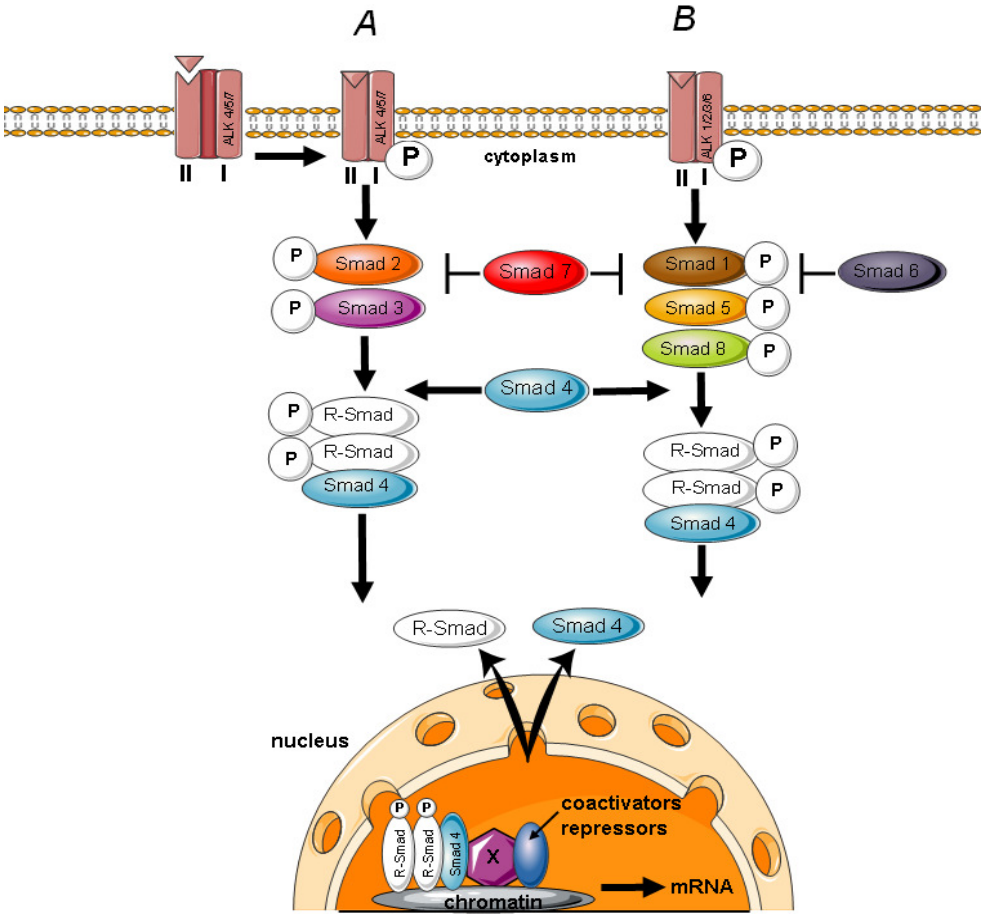

Figure 2: General mechanism of the (A) TGF-β and activin/nodal and (B) BMP pathways, with their corresponding Smad proteins. The ligand binds a complex of transmembrane receptor serine/threonine kinases (type I and II). The activated type I receptors phosphorylate selected Smads, and these receptor-activated Smads (R-Smads) then form a trimer of two R-Smads and a single Smad4, which is subsequently translocated into the nucleus. Nuclear Smad complexes then regulate transcription of target genes, through physical interaction and functional cooperation with DNA-binding transcription factors (X) and coactivators or repressors. Activation of R-Smads by type I receptor kinases is inhibited by Smad6 or Smad7.

This classical view of the TGF-β superfamily member signaling pathways is complicated by the fact that TGF-β family members can also signal through Smad independent signaling routes, involving several members of the mitogen-activated protein kinase (MAPK), Akt and RhoA signaling cascades (Figure 3). As these pathways are also capable of cross-talk, the signaling cascade of TGF-β superfamily members becomes a complex network.

Although TGF-β1 is an important player in this network, increased levels of TGF-β1 do not always condemn the kidney to fibrosis, as illustrated by BMP-7 treatment preventing renal fibrosis despite elevated levels of TGF-β1[16;24]. Therefore, research addressing the involvement of other TGF-β family members in renal fibrosis may provide new therapeutic targets for treating fibrosis in diabetic nephropathy.

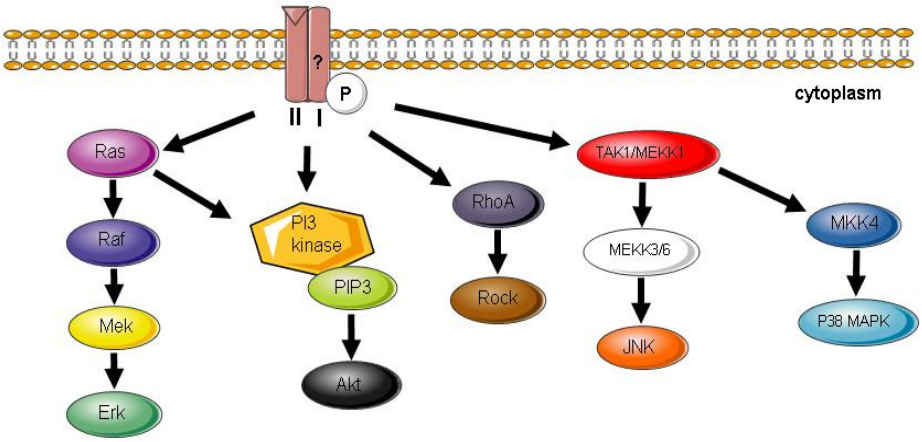

Figure 3: TGF-β receptor signaling through Smad-independent pathways. Non-Smad signaling pathways downstream via the TGF-β receptors.

Growth Differentiation Factor 15 (GDF15)

In a microarray study described in chapter 2, we identified the TGF-β family member, Growth Differentiation Factor 15 (GDF15), to be highly upregulated in kidney after induction of experimental diabetes. The upregulation of GDF15 preceded changes in TGF-β1 expression, despite early activation of renal fibroblasts. In addition, epidemiological data became available suggesting a link between circulating GDF15 levels and the incidence of cardiovascular disease and diabetes[28]. We therefore hypothesized that GDF15 is a factor involved in the development of diabetic nephropathy.

GDF15 is also known as macrophage inhibitory cytokine-1 (MIC-1), placental transforming growth factor-β (PTGF-β) or β-N-acetylglucosaminidase-1 (NAG-1). Previous studies demonstrated that GDF15 is involved in the regulation of several cellular processes, including the cell cycle[29], proliferation[30], differentiation[31] and apoptosis[32;33]. GDF15 is generated as a 40-kDa propeptide and after cleavage of the N-terminus, a 30-kDa disulfide-linked dimeric protein is secreted as the active form[34]. Unlike other TGF-β family members, the propeptide of GDF15 is not required for proper folding and secretion[35].

In healthy animals, GDF15 is expressed in the placenta and the prostate with low expression in other organs. However, GDF15 is highly expressed as an immediate-early gene in multiple organs after a wide variety of injuries. Cardiomyocytes express and secrete GDF15 during periods of ischemia and reperfusion[36]. As GDF15 knockout mice develop more cardial damage, GDF15 was suggested to be a protective factor[37]. Further, GDF15 was upregulated after injury in the lung, liver and kidney[38;39], suggesting that GDF15 induction is a widespread cellular injury response.

The receptor for GDF15 is still unknown. Several reports document activation of TGF-β responsive promoters and the inhibition of cellular growth by GDF15 being dependent on expression of TGF-β receptors type I and II, or Smad4[39;40]. However, based on the similarity in the primary structure, GDF15 may be closer to the BMP subfamily than to the TGF-β subfamily[41] and may therefore activate BMP receptors. In cardiomyocytes GDF15 elicited a TGF-β/ activin-like response through Smad2,3, but also activated ERK and Akt[42], suggesting that GDF15 is involved in the activation of Smad-independent pathways. Thus, GDF15 may provoke both signaling pathways that correspond or deviate from canonical TGF-β signaling, possibly depending on the cell type investigated.

Scope of this thesis

The overall aim of the studies described in this thesis is to establish the role of GDF15 and its signaling pathways in the development of diabetic nephropathy.

In *chapter 2* we explored the early changes in renal gene expression after the induction of experimental type 1 diabetes in rats. GDF15 was found to be upregulated immediately after induction of diabetes. Smad2 activation and smooth muscle actin expression were both detectable in the absence of increased TGF-β expression, suggesting that other factors than TGF-β (such as GDF15) may be involved in the development of early fibrosis.

In *chapter 3* of this thesis, the signaling cascades activated by GDF15 and the involvement of GDF15 on proliferation, apoptosis, profibrosis in the development of diabetic nephropathy were studied *in vitro* in human mesangial cells.

In *chapter 4* we investigated the effect of the genetic deletion of GDF15 on the development of diabetic nephropathy in experimental models of type I and type II diabetes in mice.

As vasomotor control has been shown imperative in the development of kidney damage, the effects of GDF15 on vasomotor responses of mice aorta were evaluated in *chapter 5*.

In *chapter 6* we explored whether blood levels of GDF15 predict the progression of nephropathy in type 2 diabetic patients and in patients with hypertension.

Finally, in *chapter 7* the main findings of the studies are summarized and discussed into a broader perspective. The implications for clinical practice and future research are presented.

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Chapter 2

Immediate activation of pre-fibrosis following the induction of experimental diabetes in rat: a microarray analysis of genes involved

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Azuwerus van Buiten
Attje Hoekstra
Maria Sandovici
Leo E. Deelman

Abstract

Background

Experimental diabetic nephropathy is a chronic kidney disease characterized by the development of tubulointerstitial and glomerular fibrosis several weeks after the onset of diabetes. The pathways leading to fibrosis are apparently initiated early after induction of diabetes, as pre-fibrotic markers are detectable within one week of diabetes. In the current study, we aimed at identifying genes associated with renal pre-fibrosis in the first week after induction of diabetes in the rat streptozotocin (STZ) model.

Methods

Male Wistar rats were made diabetic by a single injection of STZ. mRNA was isolated from kidneys at days 1, 2, 4 and 7 after STZ induction. An expression array including dye swap was performed at all time points and expression of individual genes within the first week of diabetes were compared to non-diabetic controls. The area under the curve was calculated to quantify the overall level of changes in expression of individual genes.

Results

Blood glucose levels were elevated at day one and remained similarly elevated at all subsequent time points. Induction of renal pre-fibrosis was confirmed by increased α-SMA expression and increased phosphorylated-Smad2/3 levels after one day 1 of diabetes and at all later time points. A total of 290 genes were found to be significantly changed in the first week of diabetes. Initiation of renal pre-fibrosis was not associated with increased renal expression of TGF-ß mRNA expression but changes in the expression of several modulators of TGF-ß signaling including GDF15, one of the most prominent genes up-regulated early in the diabetic injury. Moreover, P311, follistatin and two proprotein activating proteases showed prominent expression changes, suggesting the involvement of these factors in the induction of pre-fibrosis in early diabetes.

Conclusion

We identified fibrosis related genes in rat kidney that are upregulated shortly after the induction of diabetes. Previously, some of the identified genes have been associated with a more advanced stage of renal fibrosis. Our study suggests that these genes may be involved in the initiation of pre-fibrosis in early diabetes. These genes and their signaling pathways may represent targets to interfere in early diabetic nephropathy.

Introduction

In the near future, a dramatic increase in the prevalence of diabetes mellitus is expected[1;2]. Despite glycemic control, long lasting diabetes can lead to complications including diabetic retinopathy, diabetic neuropathy, and diabetic nephropathy in both type I and type II diabetes. Control of blood glucose levels through various drugs, including alpha-glucosidase inhibitors, amylin analogues, DPP-4 inhibitors, glinides, GLP-1 derivatives and and insulins[3] has proven to attenuate these complications[4;5]. Furthermore, reduction of blood-pressure through the use of inhibitors of the Renin-Angiotensin System (RAS) has proven beneficial in the prevention of diabetic nephropathy[6], and evidence suggests this effect to be in part mediated by inhibited renal expression of the fibrogenic cytokine TGF-β[7] .

Diabetic nephropathy is a progressive chronic kidney disease and on average, patients develop diabetic nephropathy after 15 – 20 years of diabetes. Both types of diabetes are associated with the development of diabetic nephropathy, a condition that ultimately progresses into end-stage renal disease. Despite the benefits of blood sugar and blood pressure control, current therapies do not halt the progression of diabetic nephropathy[8].
Several animal models of diabetes have been developed[9;10] and although the underlying cause of diabetes differs between models, diabetic nephropathy usually develops much faster in experimental models than in humans. In rodents, renal fibrosis can be detected after several weeks of diabetes[11]. Nevertheless, similar histological changes are present in kidneys of both diabetic humans and animals, including glomerular and tubular basement membrane thickening, glomerulotubular hypertrophy/ hyperplasia, mesangial and tubulointerstitial expansion (extracellular matrix [ECM] accumulation), glomerulosclerosis and tubulointestinal fibrosis[12], indicating that animal models may be used to investigate the molecular pathways leading to diabetic nephropathy.

Experimental models of diabetes show that differentiation of interstitial fibroblasts into myofibroblasts is one of the initiating mechanisms leading to diabetic nephropathy[13]. Once activated, myofibroblasts produce extracellular matrix molecules including collagens, proteoglycans and fibronectin as well as proteases capable of degrading the ECM[14]. These changes contribute to the development of renal interstitial fibrosis, a key feature of diabetic nephropathy.

The differentiation of renal fibroblasts into myofibroblasts seems to represent a very early event in diabetes and is already present after 7 days in the streptozotocin (STZ) induced diabetic rat[15]. As activation of myofibroblasts is a common feature in several models of kidney disease, unraveling the mechanisms of early myofibroblast activation in diabetes, may therefore contribute to a better understanding of the development of kidney fibrosis in general.

In this study, we aimed at identifying factors that are associated with the activation of fibrosis within the first week of diabetes in the rat single dose STZ model. To this end, we performed an expression microarray analysis on rat kidneys obtained on 0, 1, 2, 4 and 7 days after the induction of diabetes. Gene expression timecurves were constructed relative to non-diabetic controls. The area under the curve was calculated to quantify the expression of genes that were selectively up- or down-regulated in the first week of diabetes. Although expression microarrays have been performed previously in the rat STZ model[16;17], this is the first time that renal gene expression was characterized at these early timepoints after the induction of diabetes.

Materials and methods

Animals

Male Wistar rats (weight 250 – 270 g, 7-8 weeks old, Harlan) were used in the study. The animals were acclimatized to the environment for at least 3 weeks prior to experimentation. All experimental procedures were conducted according to the guidelines for the use of experimental animals approved by the Animal Experiments Ethical Committee of the University of Groningen, the Netherlands. Animals were housed under standard conditions with free access to food (standard rat chow) and drinking water throughout the study. Diabetes was induced in rats by i.v. injection of streptozotocin (STZ) in citrate buffer (60 mg/kg). Blood samples were taken before induction for measurement of glucose levels. A sham group injected with citrate buffer only (n=10) was included. STZ treated rats were sacrificed at day 1 (n=6), 2 (n=7), 4 (n=5) and 7 (n=6) after induction of diabetes. At sacrification, rats were anesthetized with isoflurane (2%) and an aortic blood sample was collected. Plasma was isolated and stored at -80˚C. The kidneys were perfused with saline and removed. Midcoronal slices were fixed in 4 % paraformaldehyde, processed for paraffin embedding and further used for immunohistochemistry. A second slice was frozen in liquid nitrogen, stored at -80˚C and used for RNA isolation.

RNA isolation

Total RNA was isolated with a Qiagen RNA isolation kit (Qiagen, Venlo, The Netherlands) from rat frozen kidney samples containing both cortex and medulla. The RNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 nm. RNA integrity was checked on 1% agarose gels.

Messenger-RNA amplification and Cy-dye coupling

Linear amplification of mRNA was performed according to a protocol of the Dutch Cancer Institute (www.nki.l.nl/nkidep/pa/microarray/protocols.htm). Briefly, amplification started with first strand cDNA synthesis from 2 µg of total RNA, using Superscript II RT-polymerase (GIBCO - Invitrogen Inc, Carlsbad, Ca, USA) and a specific oligo(dT) primer containing a 17bp T7 polymerase recognition site (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG[T]24-3') (Eurogentec, Seraing, Belgium). After second strand synthesis, double-stranded cDNA was purified with the Qiaquick PCR purification kit (Qiagen) and the yield was determined spectrophotometrically. *In vitro* transcription was performed with the T7 Megascript kit (Ambion, Huntingdon-Cambridgeshire, UK) as described by the manufacturer and aminoallyl-UTP (Ambion) was incorporated as described by Hoen et al (2003). Amplified RNA (aRNA) was purified with the RNA clean up protocol (Qiagen). Five µg of aRNA was labeled by coupling monoreactive Cyanine-3 or Cyanine-5 fluorophores (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) to the aminoallyl-modified nucleotides. Labeled aRNA was separated from unincorporated Cyanine-3 or Cyanine-5 molecules over Microspin G50 columns (Amersham Biosciences) as described by the manufacturer.

For the identification of genes differentially expressed between diabetic animals and non-diabetic control, a common reference design was applied. In this design, day 0 (non-diabetic, Cy3 labeled) was compared to each consecutive time point (days 1, 2, 4 and 7, Cy5 labeled), resulting in 4 arrays. In addition, a dye swap was performed, resulting in a total of 8 array hybridizations. For each time point, equal amounts of total RNA of at least five animals were pooled.

Micro-array slides and hybridization

Operon OpArray rat slides V3 printed with 26962 oligos were purchased from Operon Biotechnologies, Germany. Blocking, prehybridization and hybridization were performed as described by Hegde et al. (2000) with some modifications. In short, slides were blocked

with ethanolamine at 42°C during 1 h. Prehybridization was performed with prewarmed prehybridization buffer containing 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) at 42°C during 45 min. Subsequently, the slides were washed three times with preheated water (52°C), dried by centrifugation at 620 rpm during 5 min and immediately used for hybridization. The hybridization sample consisted of the fluorescently labeled probe mixture and 30 µg poly-A (Sigma Aldrich) mixed with an equal volume of preheated (52°C) two times concentrated hybridization buffer. This hybridization sample was heated at 95°C for 3 min before it was applied to the preheated slides. Hybridization was performed in hybridization chambers (Telechem International Inc, Sunnyvale, CA, USA) in a water bath at 42°C in the dark for approximately 48 h. Subsequently, slides were washed with 5 wash solutions under agitation: 1xSSC/0.2% SDS at 42ºC; 0.1xSSC/0.2% SDS at 42ºC; 0.1xSSC at 42º; 0.1xSSC at room temperature and 0.01xSSC at room temperature. Each wash step lasted 5 minutes. Finally, slides were dried by centrifugation at 600 rpm during 5 min and scanned with an Affymetrix GMS428TM array scanner.

Micro-array data analysis

Fluorescent signal intensities from scanned images were processed and analyzed using Agilent Feature Extraction software version 9.1.3.1 (Agilent Technologies). A quality control report was generated for each array. Data was imported into GeneSpring GX version 7.3.1 (Agilent, Santa Clara, CA, USA). This software package allows multi-filter comparisons using data from different experiments to perform the normalization, generation of restriction (filtered) lists and functional classifications of the differentially expressed genes[18]. Normalization was applied in dye swap in three steps: 'per spot normalization' divided by control channel; 'per chip normalization', where each measurement was divided by the 50th percentile of all measurements in its array; and 'per gene normalization', by which all the samples were normalized against the median of the control samples. After normalization, the expression of each gene was reported as the ratio of the value obtained for each condition differential to the control condition. As each slide was also performed with a dye swap, the gene expression values from both arrays were averaged.

Plots were constructed to evaluate the relative expression of individual genes in time. To identify genes that are specifically regulated within the first week after induction of diabetes, we calculated the area under the curve (AUC) for individual genes on the array using the trapezoid method. Differences in time ranges between different experimental days

were accounted for in the AUC calculation. Genes were considered significantly regulated if their AUC was outside the 3 standard deviation limit.
This method does not recognize significant up- or downregulation of a gene at specific time points, but rather provides a measure to determine the extent of change in relative gene expression of a particular gene within the first week of diabetes. Typically, spots with low intensities provide noise and therefore these spots are usually filtered out by setting a threshold for signal intensity. In our area under the curve calculation, negative areas (down-regulation) compensated for positive areas (up-regulation), further eliminates noise. Therefore all spots that passed the quality control check were included in the analysis.

RT PCR

PCR analysis was employed to verify the results of selected array data with real-time PCR. For this, 1µg of kidney RNA of individual rats was reversely transcribed into cDNA. Real-Time PCR samples were prepared with 2× ABsolute™ QPCR SYBR® Green ROX Mix (Westburg, Leusden, Netherlands), 10 uM of primer and 2.5 µl template DNA in a total volume of 25 µl. The PCR profile consisted of 15 min at 94 °C, followed by 40 cycles with heating to 94 °C for 15 s and cooling to 60 °C for 1 min. PCR product specificity and purity was evaluated by generating a dissociation curve following the manufacturer's recommendations. The sequences of the primers used are listed in Table 1.

Table 1: Primer sequences

gene	Forward primer (5'→3')	Reverse primer (5'→3')
TGF-ß1	AACAATTCCTGGCGTTACCT	TATTCCGTCTCCTTGGTTCA
vimentin	CTCTGGCACGTCTTGACCTT	CTGCACCTGTCTCCGGTATT
collagen 3A1	AAGGACACGCTGGTGCTCAA	TTCGCCTGAAGGACCTCGTT
GDF15	ACCTGCTGAGCCGACTGCAT	CCAATCGCACCTCTGGACTG
P311	CCTGTGCCTAAGGAAGTGA	GGTGGAGGTAACCGATAGC
follistatin	CAGTGCCTGCCACCTGAGAA	CAGCGACCTCTGCCAACCTT
KIM-1	GTCTGTATTGTTGCCGAGTG	GGTCTTGTTGGAGGACTTGT
PACE4	CACCGCAGGCAGAGACTCCA	CACGGCGACAAGACAGGCAC
aminopeptidase O	GCCATCAGGATATGGTATAA	TGCTCAGTCAGATCATCTAA
KAP	TCTAATGGAGCTGAGACAGT	AGGAGGTAGAGGAGAATGTA

Western blotting

Frozen kidney fragments were crushed and lysed in 300 µl of ice-cold RIPA buffer (1% Igepal ca-630, 1% SDS, 5 mg/ml sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM ß-mercapto-ethanol, 40 µg/ml PMSF, 100 µg/ml benzamidine, 500 ng/ml pepstatin A, 500 ng/ml leupeptine and 500 ng/ml aprotinin in PBS). Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, The Netherlands). On a 5-20% SDS-PAGE gel, 20 µg of total protein was run and transferred to nitrocellulose. Phosphorylation of Smad2/3 was detected with the p-Smad2/3 (Ser 423/425) antibody (sc-11769, Santa Cruz) in combination with a polyclonal HRP conjugated anti-goat IgG secondary antibody (sc-2768, Santa Cruz). GDF-15 expression was detected with the primary antibody Mic-1 (Y60) (Cell Signaling Technology, #3249S) in combination with polyclonal HRP conjugated goat anti-rabbit IgG secondary antibody (P0448, Dako) and a housekeeping monoclonal GAPDH antibody (10R-6109a) with a polyclonal HRP conjugated anti-mouse IgG secondary antibody (P0260, Dako) was used. Signals were detected by the ECL-detection method (Amersham, The Netherlands).

Immunohistochemistry

To evaluate renal prefibrosis after induction of diabetes, sections were stained for α-smooth muscle actin (mouse monoclonal anti-α- smooth muscle actin, α-SMA, Sigma Chemical Co, St Louis, MO, USA). Before the immunostaining procedure, paraffin sections (3 µm) were dewaxed and subjected to antigen retrieval by an overnight incubation in 0.1M Tris/HCl buffer, pH 9.0, at 80º. For immunohistochemistry, a two-step immunoperoxidase technique was used, according to standard methods, as previously described[19]. Peroxidase activity was developed using 3',3'-diaminobenzidine tetrachloride and H_2O_2. The expression of α-SMA by immunohistochemistry was measured using computer-assisted morphometry. Totals of 30 fields were evaluated at a magnification of 200×. The total area of staining (excluding blood vessels and glomeruli) was divided by the total area measured and expressed as a percentage.

TGF-β bioassay

Active and latent TGF-β levels were determined as described previously[20]. In short, Mv1Lu cells stably transformed with a SBE-luciferase reporter construct[21], were plated in 96 wells plates. Single wells were incubated overnight with 50 ul DMEM and 50 ul heparinized plasma. Latent TGF-β was activated by adding HCl (80 mmol/L) to the plasma and incubation for 1 hour at 4°C. Thereafter, the solution was neutralized by the addition of

NaOH (80 mmol/L) to the plasma. Luciferase activity was determined using Promega's luciferase assay system according to the manufacture's protocol.

Statistical analysis

Data are expressed as mean value ± S.E.M. Differences between groups were tested by Student's t-test and analysis of variance (ANOVA) by Microsoft Excel (2003 SP3 version). The normal distribution of the AUC was tested with a Kolmogorov-Smirnov test with Lilliefors Significance Correction using SPSS 16. A p value less than 0.05 was considered to be statistically significant.

Results

Induction of diabetes

In STZ injected rats, blood glucose levels were elevated as of day 1 and remained at similar levels at all subsequent time points (Table 2). Furthermore, diabetic animals showed decreased body weights at all time points.

Table 2: Main animal characteristics

	control	diabetic			
		day 1	day 2	day 4	day 7
Body weight (g)	378 ± 12.4	340 ± 17.2*	322 ± 13*	269 ± 9.6*	329 ± 11.5*
Glucose (mM)	8.5 ± 0.5	23 ± 2.5*	23.6 ± 1.7*	19.3 ± 1.9*	24.8 ± 1.9*
L kidney (g)	1.8 ± 0.1	1.6 ± 0.1	1.9 ± 0.1	1.7 ± 0.1	1.9 ± 0.1
R kidney (g)	1.4 ± 0.04	1.4 ± 0.05	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.2

Values are mean ± SEM (* $p<0.05$ versus control)

Markers of renal pre-fibrosis

Renal pre-fibrosis was quantified by measuring α-SMA staining in the cortical interstitium. α-SMA was significantly increased on day 2 (Figure 1*A*) and remained elevated at all subsequent time points. Further, activation of the profibrotic Smad2/3 pathway was determined by measuring the levels of phosphorylated Smad2/3 in kidney homogenates by western-blot. Phosphorylated-Smad2/3 levels were elevated after the induction of diabetes as of day 1 onwards (Figure 1*B*). Real-time PCR for TGF-ß1, vimentin, collagen 3A1 and KIM-1 did not show increased expression at any of the time points (Figure 1, panels *C,D,E* and *F*).

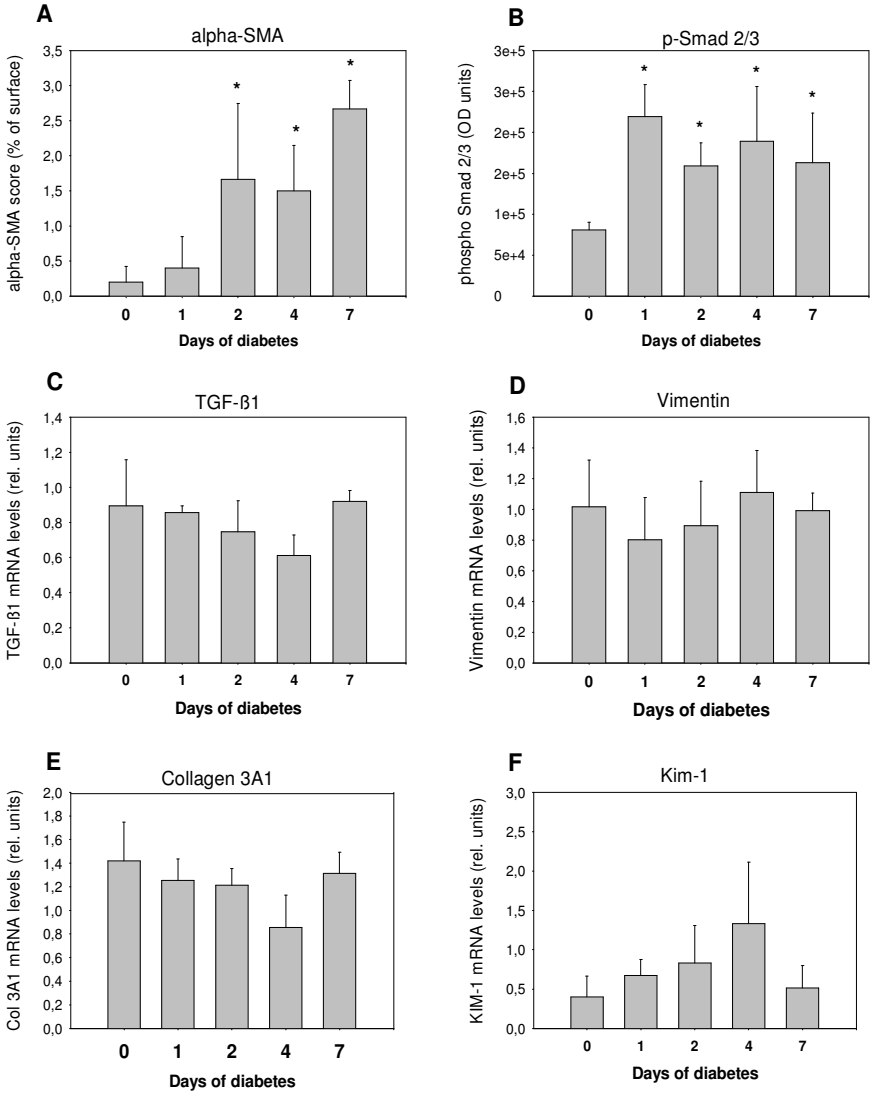

Figure 1: Expression of pre-fibrotic markers after STZ mediated induction of diabetes

A) Quantification of interstitial alpha-SMA expression on stained immunohistochemical sections. B) Quantification of Smad 2/3 phosphorylation by western blot using a phospho-specific Smad2/3 antibody. C,D,E and F) Real-time PCR quantification of renal TGF-ß1, vimentin, collagen 3A1, KIM-1 mRNA expression. * indicates significantly different ($P<0.05$) from day 0.

Latent and active TGF-ß levels in plasma

Mink lung cells stably transfected with a TGF-β sensitive SBE-luciferase reporter construct were incubated with untreated or acid activated plasma of diabetic animals for 24 hours. At day 1, active TGF-ß was moderately decreased (Figure 2*A*) while latent TGF-ß was increased more than 20-fold (Figure 2*B*). Levels of active and latent TGF-ß returned to control values by day 2 and subsequent time points.

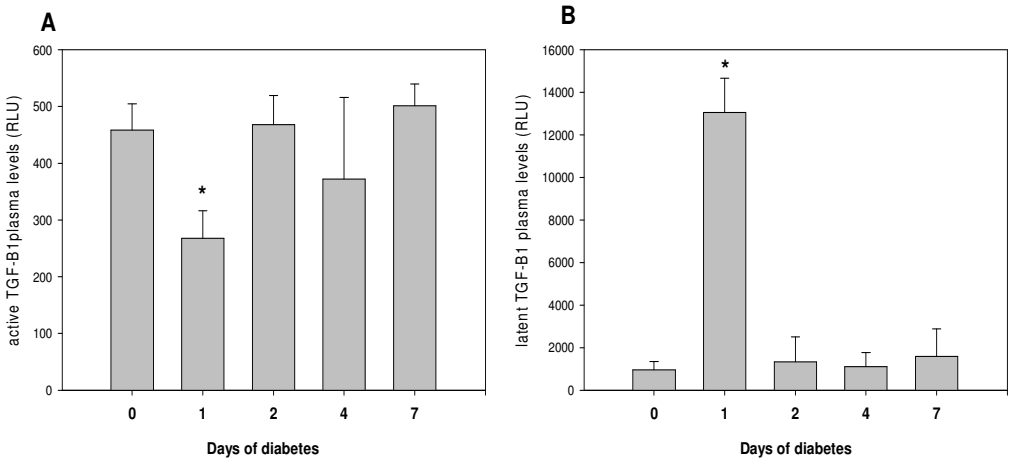

Figure 2: Levels of active and latent TGF-β1 in the plasma of diabetic animals

A) Active TGF-β1 levels were decreased at day 1 and normalized by day 2. B) Latent TGF-β1 levels were increased by day 1 and normalized by day 2 (* indicates $p<0.05$ vs day 0).

Gene expression analysis

The Operon OpArray rat slides contained 26.989 oligonucleotide spots. After hybridization, spots were scanned and typically more than 99.9% of the spots on each slide passed the quality control tests for spot shape and low spot background outside of the spot. After quantification of the arrays, plots were constructed to evaluate the relative expression of individual genes in time. To identify genes that were specifically regulated within the first week after induction of diabetes, we calculated the area under the curve (AUC) for individual genes on the array using the trapezoid method (Figure 3*A*). A histogram plot of the AUC's of gene expression in time (Figure 3*B*) demonstrated a normal distribution ($p<0.001$). We considered genes with AUC's outside 3 times the standard deviation from the mean as being significantly changed during the first week of diabetes. The mean in AUC amounted to 0.009 ± 0.395 for the 26989 genes investigated. Thus, genes outside the

range of -1.176 to 1.194 fold change were considered significantly changed. In this way, 290 genes were identified to be specifically regulated within the first week of diabetes (see Table 3 for the top 25 up- and down regulated genes).

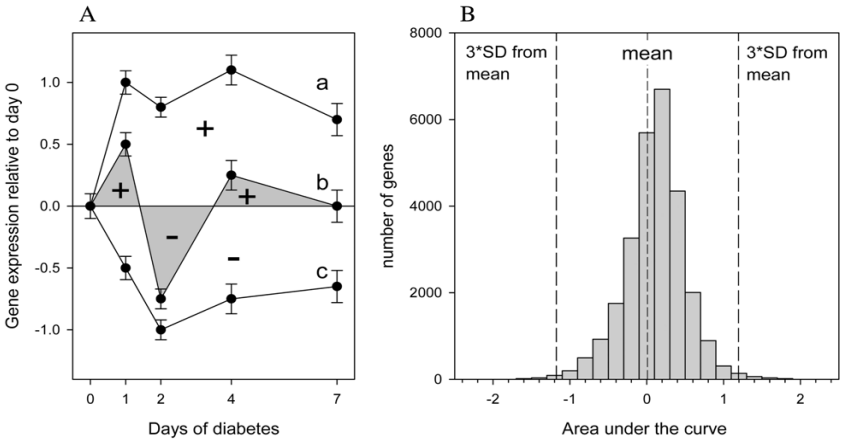

Figure 3: Normal distribution of the area under the curve (AUC) for gene expression in time

A) Examples for calculation of the AUC. Relative expression of individual genes (log(ratio)) compared to non-diabetic controls (day 0) were plotted in time. Example plots a and c demonstrate up regulated and down regulated genes, respectively. Plot b: Positive and negative areas were not converted to absolute numbers, resulting in a sum of the positive and negative areas that is close to zero, eliminating genes that show erratic expression in time. B) The AUC was calculated for all individual genes and a histogram plot was constructed to visualize the distribution of the AUC of gene expression in time. The AUC's demonstrated a normal distribution ($p<0.001$) with a mean of 0.009. Genes with AUC's outside 3 standard deviations of the mean were considered significantly different from non diabetic controls.

Gene ontology

As we aimed at identifying factors which are associated with the activation of myofibroblasts within the first week of diabetes, we identified all factors associated with TGF-ß signaling, cell growth, differentiation and proliferation, apoptosis, response to stress and protease activity in the list of 290 genes (Table 4).

Renal upregulation of Growth Differentiation Factor 15 (GDF15)

GDF15, a member of the TGF-ß family was the second most strongly up-regulated gene in our array analysis. The upregulation of GDF15 in the first week of diabetes was confirmed with RT-PCR on individual animals (Figure 4A), demonstrating a more than 20-fold

induction of GDF15 mRNA expression on days 1 and 2 after induction of diabetes. GDF15 expression showed a trend towards normalization to control levels at subsequent time points, although GDF15 levels were still significantly increased at day 7. In addition, protein levels of renal GDF15 expression approved RT-PCR confirmation (Figure 4B).

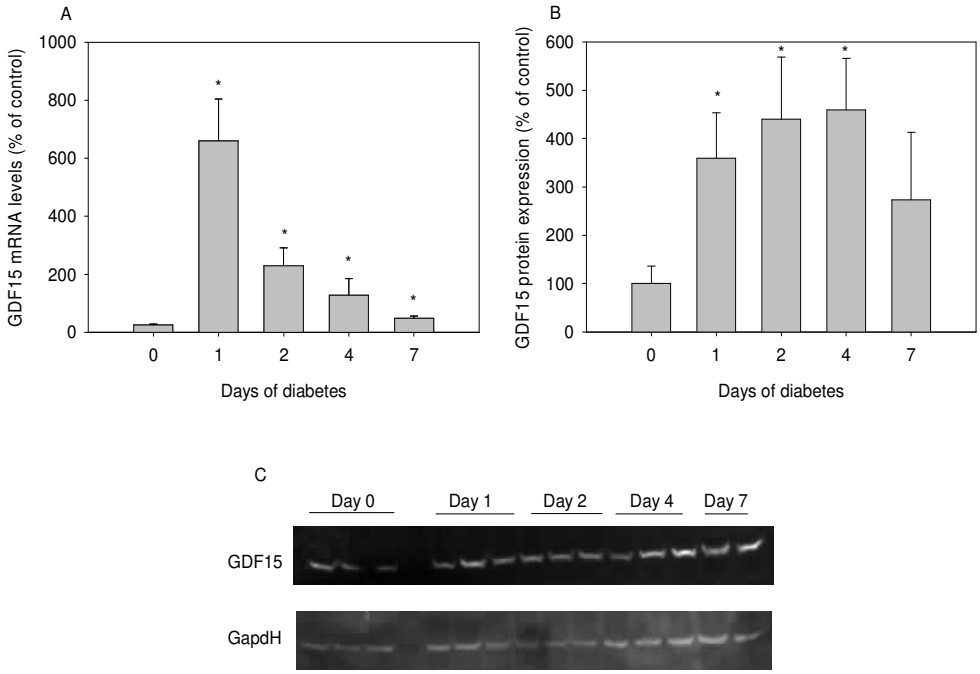

Figure 4: Up-regulation of renal GDF15 expression

A) Real-time PCR on kidneys of individual rats confirms the up-regulation of renal GDF15 mRNA expression after induction of diabetes. B) GDF15 protein levels by western blot. C) Representative picture shows GDF15 protein expression from 2-3 samples per group. The blot was re-probed for GapdH to show equal loading Final data from 2 blots are means ± SEM (* indicates P< 0,05 vs day 0)

Confirmation of array data by real-time PCR

To confirm the results obtained with the microarray, real-time PCR was performed for a subset of genes related to renal prefibrosis (Figure 5). Real-time PCR on individual kidneys within each group showed similar patterns in gene expression as with the microarray technique.

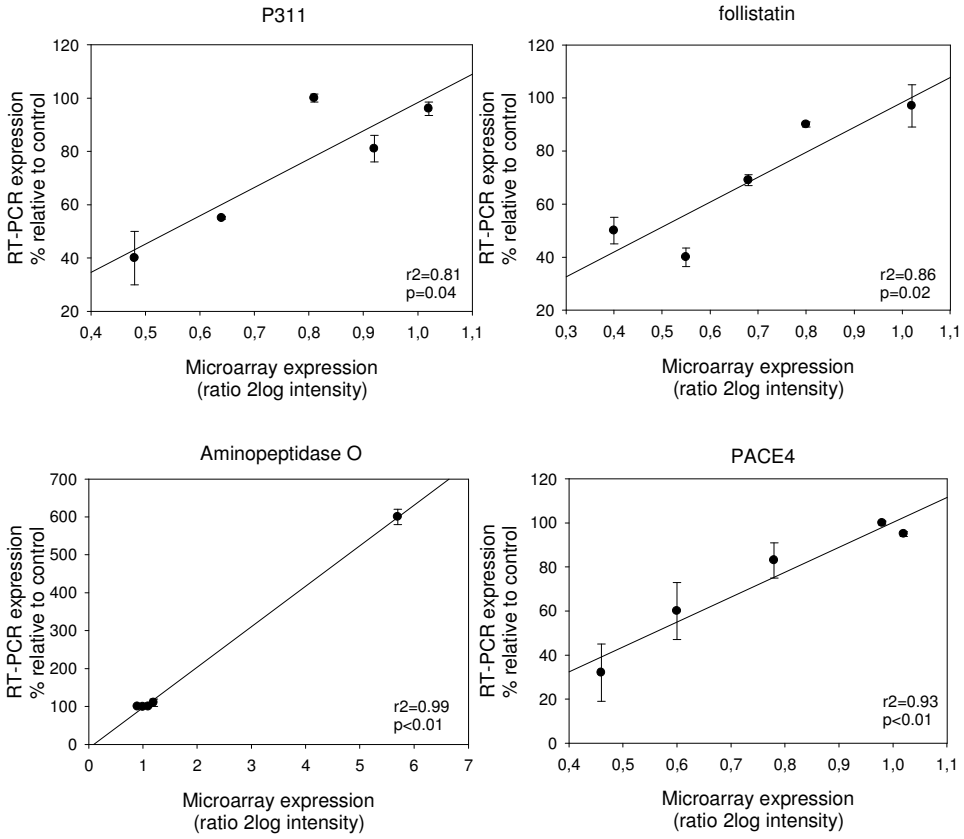

Figure 5: Confirmation of microarray gene expression and real-time PCR expression

Table 3: Gene list showing the top 25 up and down regulated renal genes in the first week after induction of diabetes

AUC	Gene acc. #	Description
Top 25 up regulated genes		
2.76	NM_173323	UDP-glucuronosyltransferase
2.28	NM_019216	growth differentiation factor 15
2.26	NM_173094	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
2.23	XM_216142	retinoic acid receptor responder (tazarotene induced) 2
2.19	NM_053587	S100 calcium binding protein A9 (calgranulin B)
2.09	XM_343171	Rattus norvegicus lymphoctye Met-ase 1 (Gzmm), mRNA
2.01	NM_201418	Ns5atp9 protein
1.95	NM_183403	Rattus norvegicus glutathione peroxidase 2 (Gpx2), mRNA.
1.92	XM_241671	similar to DNA2 DNA replication helicase 2-like
1.87	NM_144755	tribbles homolog 3
1.87	NM_012716	solute carrier family 16 (monocarboxylic acid transporters)
1.86	XM_346212;XM_228626	similar to Discs large homolog
1.81	NM_031834	sulfotransferase family 1A, phenol-preferring, member 1
1.80	XM_228669;XM_229139	high-mobility group box 4
1.77	NM_012559	fibrinogen, gamma polypeptide
1.75	XM_238283	similar to Glycerol-3-phosphate acyltransferase
1.74	NM_053822	Rattus norvegicus S100 calcium binding protein A8 (S100a8)
1.73	NM_175763	immunoglobulin superfamily, member 1
1.71	NM_023980	G protein-coupled receptor 74
1.70	XM_215293	vanin 1
1.70	M84148	Rat IgK chain VJ1 region mRNA
1.69	NM_019296	cell division cycle 2 homolog A
1.69	XM_224970	similar to cytoskeleton associated protein 2
1.66	NM_199115	angiopoietin-like protein 4
1.65	NM_201635;XM_230882	cytochrome P450, subfamily 24
Top 25 down regulated genes		
-4.200	NM_019347	UREA TRANSPORTER, KIDNEY.
-2.678	NM_052802	KIDNEY ANDROGEN-REGULATED PROTEIN.
-2.445	XM_342237	PDZ domain containing 6 (predicted)
-2.214	NM_012784	Rattus norvegicus cannabinoid receptor 1 (brain) (Cnr1), mRNA
-2.073	NM_001011948	Rattus norvegicus solute carrier family 7 (cationic amino acid
-2.014	NM_001017465	Rattus norvegicus similar to solute carrier family 7
-1.994	NM_001000851	olfactory receptor 807 (predicted)
-1.970	NM_031044	HISTAMINE N-METHYLTRANSFERASE (EC 2.1.1.8) (HMT)
-1.866	NM_030837	SOLUTE CARRIER FAMILY 21 MEMBER 4
-1.848	XM_001070678	PREDICTED: Rattus norvegicus similar to taste receptor, type 2
-1.830	XM_345911	Similar to BMP-binding endothelial regulator
-1.806		unknown EST
-1.801	NM_001077650	Rattus norvegicus endothelin 3 (Edn3), mRNA
-1.791		unknown EST
-1.770	XM_234288	sine oculis-related homeobox 6 homolog (Drosophila) (predicted)
-1.746	NM_001008944	vomeronasal 1 receptor, j4
-1.745	XM_001078113	PREDICTED: Rattus norvegicus AHNAK nucleoprotein
-1.722	NM_019169	ALPHA-SYNUCLEIN.
-1.696	NM_001106778	Rattus norvegicus anoctamin 4 (Ano4), mRNA
-1.674	XM_216331	similar to hypothetical protein (predicted)
-1.673	XM_229020	similar to dystrophin
-1.650	NM_012566	ZINC FINGER PROTEIN GFI-1
-1.641	NM_001000548	olfactory receptor 158 (predicted)
-1.640	XM_218181	NACHT, leucine rich repeat and PYD containing 12 (predicted)
-1.615	XM_219775	Rattus norvegicus similar to PD-1-ligand precursor

Table 4: List of genes involved in TGF-ß signaling, cell growth, differentiation and proliferation, apoptosis, response to stress and protease activity which were up or down regulated within the first week of diabetes. Genes were grouped based on gene ontology classification numbers provided with the operon microarray slides.

AUC	Gene acc. #	Description
7179 (transforming growth factor beta receptor signaling pathways), 17015 (regulation of transforming growth factor beta receptor signaling pathway)		
2.28	NM_019216	growth differentiation factor 15
-1.20	NM_178096	neuronal regeneration related protein P311
0008151:cell growth and/or maintenance		
1.57	NM_012930	carnitine palmitoyltransferase 2
1.42	NM_012879	solute carrier family 2 (facilitated glucose transporter), member 2
1.38	NM_130741	lipocalin 2
1.37	NM_031821	polo-like kinase 2
1.32	XM_220119;NM_001009470	cyclin B2
1.32	XM_219276	similar to ATP-binding cassette transporter sub-family A member 15
1.32	NM_017100	polo-like kinase 1
1.26	NM_080782	cyclin-dependent kinase inhibitor 1A
1.25	XM_220540	similar to solute carrier family 5 (sodium/glucose cotransporter), member 10
-1.32	NM_031584	solute carrier family 22 (organic cation transporter), member 2
-1.40	NM_012488	alpha-2-macroglobulin
-1.44	XM_223975	similar to Zinc transporter ZIP2 (Eti-1) (6A1) (hZIP2) (Solute carrier family 39 member 2)
-4.20	NM_019347	solute carrier family 14 (urea transporter), member 2 isoform 1
5172 (vascular endothelial growth factor receptor binding), 8283 (cell proliferation), 8284 (positive regulation of cell proliferation), 8285 (negative regulation of cell proliferation), 30154 (cell differentiation), 42127 (regulation of cell proliferation), 45596 (negative regulation of cell differentiation),		
1.66	NM_199115	angiopoietin-like protein 4
1.49	XM_215849	similar to budding uninhibited by benzimidazoles 1 homolog
1.29	XM_215924	similar to ubiquitin-conjugating enzyme E2C
1.22	XM_225460	similar to Antigen KI-67
-1.21	XM_215890	similar to Angiopoietin-4 precursor (ANG-4) (ANG-3)
-1.35	NM_030827	low density lipoprotein receptor-related protein 2
-1.52	NM_017082	Uromodulin
-1.54	XM_343923	similar to retinoic acid, EGF, and NGF upregulated
-1.56	NM_012561	Follistatin
6915 (apoptosis), 6916 (anti-apoptosis), 6919 (caspase activation), 42981(regulation of apoptosis), 43066 (negative regulation of apoptosis), 43065 (positive regulation of apoptosis)		
1.26	NM_022622	BRCA1 associated RING domain 1
-1.30	XM_214418	FAST kinase domains 3
-1.32	NM_139258	Bcl2 modifying factor
-1.64	XM_218181	similar to PYRIN-containing APAF1-like protein 7 isoform 2
-1.72	NM_019169	synuclein, alpha
0006950:response to stress		
1.44	NM_012861	O-6-methylguanine-DNA methyltransferase
1.39	NM_183403	glutathione peroxidase 2
0019538:protein metabolism, protease activity, peptidase activity		
1.33	NM_001012346	Rattus norvegicus aminopeptidase O
-1.26	NM_012999	PAIRED BASIC AMINO ACID CLEAVING ENZYME 4 PRECURSOR (PACE4)
-1.37	XM_235070	ubiquitin specific protease 44 (predicted)
-1.22	XM_218997	similar to ubiquitin specific protease 47

Discussion

This study is the first expression array study describing renal gene expression changes in the first week of diabetes in a rat model of STZ induced diabetes. The study aimed at identifying genes associated with the induction of prefibrosis and activation of the TGF-ß signaling pathway. Rats were diabetic as of the first day after STZ administration and blood glucose levels remained similarly elevated at all subsequent time points. The elevation of blood glucose levels was associated with increased phosphorylation of renal Smad2/3, the classical signaling route of the profibrotic factor transforming growth factor beta (TGF-ß). Furthermore, tubulointerstitial α-SMA staining was significantly increased after two days of diabetes and remained elevated at subsequent time points. These results demonstrate that induction of tubulointerstitial prefibrosis in our model of uncontrolled type 1 diabetes is already evident after just two days of diabetes. Early induction of tubulointerstitial prefibrosis in the STZ induced diabetic rats was demonstrated before, although the earliest studied time point in this study was one week after STZ injection[15]. Nevertheless, this and our study demonstrates that induction of pre-fibrosis in uncontroled STZ induced diabetes is present within the first week of diabetes.

Despite increased phosphorylation of Smad2/3 and increased α-SMA staining, we did not find evidence for increased renal TGF-β expression in the first week of diabetes using either the microarray or real-time PCR technique. It is commonly accepted that local expression of TGF-ß participates in renal Smad2/3 activation and subsequent myofibroblast activation. However, there is only limited data on renal TGF-ß expression in early STZ induced diabetes. Staining for renal TGF-ß expression demonstrated increased glomerular TGF-ß expression in the glomerulus as early as ten days after STZ injection in mice[22]. Furthermore, increased TGF-ß mRNA expression was reported in both glomerular and interstitial tissues as early as three days after STZ injection in rats with severe diabetes[23]. These differences between these studies and our data may be explained by differences in rat strain and slightly lower blood glucose levels in our study. Nevertheless, our data demonstrates a marked increase in tubulointerstitial α-SMA expression and activation of the Smad2/3 route in the absence of a strong increase in TGF-ß mRNA expression, suggesting that the TGF-ß signaling cascade is modulated through other mechanisms than upregulation of renal TGF-ß1 mRNA expression in early diabetes.

Several studies demonstrated a correlation between circulating TGF-ß levels and the progression of various fibrotic diseases including diabetic nephropathy (reviewed in[24]).

In our model, analysis of circulating latent and active TGF-ß levels demonstrated a dramatic increase in latent TGF-ß levels after one day of diabetes with subsequent normalization at all later time points. The strong transient upregulation of latent-TGF-ß plasma levels immediately upon induction of diabetes has not been documented before and the source of the latent TGF-ß is unknown. Although the increase of latent TGF-ß plasma levels normalizes by day two, local conversion and activation of circulating latent TGF-ß within the diabetic kidney may have contributed to the activation of Smad2/3 at day one after STZ injection. However, the Smad2/3 pathway remained activated at all subsequent time points, suggesting that other members of the TGF-ß family may be involved in the sustained Smad2/3 activation.
In our array analysis, we therefore focused on genes that could potentially modulate the TGF-ß signaling cascade in diabetes. In our gene ontology analysis, only one gene involved in TGF-β signaling (GDF15) was found upregulated after induction of diabetes.

Growth Differentiation Factor 15 (GDF15) is one of the most prominently upregulated genes in our array analysis and is an immediate early gene and member of the TGF-ß signaling family. The kinetics of renal GDF15 expression followed a specific pattern with a 20 fold upregulation on days one and two after induction of diabetes with a trend towards normalization at subsequent time points. Previously, renal upregulation of GDF15 mRNA expression has been demonstrated in models of renal ischemia and reperfusion injury and in the 5/6 nephrectomy model with similar kinetic profiles[25]. Furthermore, GDF15 upregulation has been demonstrated after chemical and hyperoxic injury of the lung, by surgical, chemical and heat induced injury of the liver[26] and after ischemia/ reperfusion injury of the heart[27], indicating that GDF15 is a general mediator of the organ injury response. Nevertheless, our study is the first to show that GDF15 is upregulated in the kidney immediately after induction of experimental diabetes. Furthermore, the clinical importance of GDF15 in diabetes has recently been demonstrated in a study showing that higher plasma levels of GDF15 are a predictor of all-cause and cardiovascular mortality and morbidity in patients with diabetic nephropathy[28]. Even so, the function of GDF15 in the kidney remains unknown, but studies performed in the heart and in cultured cardiomyocytes demonstrate that GDF15 has anti-apoptotic effects[29]. In addition, these studies report conflicting data on the hypertrophic effects of GDF15. Xu et al[30], demonstrated an anti-hypertrophic effect of GDF15 associated with Smad2/3 activation in the mouse myocardium and in cultured cardiomyocytes. In contrast, a recent study by

Heger et al[29] demonstrated prohypertrophic effect of GDF15 associated with Erk and subsequent Smad1 activation in cultured adult cardiomyocytes.

Furthermore, we observed down-regulation of an inhibitor of the TGF-β signaling cascades after induction of diabetes. Neuronal regeneration related peptide P311 was significantly down-regulated in the first week of diabetes. Recently, a role for P311 has been suggested in the regulation of the phenotype of myofibroblasts in immunoglobulin-A nephropathy[31]. Furthermore, a study performed in NIH 3T3 cells demonstrated that P311 stimulated smooth muscle specific protein overexpression resulted in reduced Smad3 activity, down regulated the expression of TGF-ß1 and TGF-ß2 and induced a nonfibrogenic myofibroblast phenotype[32]. Therefore, down regulation of P311 may enhance TGF-ß signaling and contribute to the development of a more pro-fibrotic myofibroblast phenotype in early diabetes.

As we found only two genes related to TGF-β signaling, we extended our analysis to factors, that may be indirectly influencing TGF-β signaling. Myofibroblast activation may be stimulated by the observed renal down-regulation of follistatin. Follistatin is an antagonist of activin A, a TGF-β family member and a potent activator of renal interstitial fibroblasts in diabetic nephropathy[33]. Local activation of latent-TGF-ß through increased protease and peptidase activity could contribute to the activation of the TGF-ß signaling cascade. We found upregulation of aminopeptidase O, an aminopeptidase related to aminopeptidase B and Leukotriene A4 hydrolase[34]. Although the involvement of aminopeptidase O is fibroblast activation are unclear, inhibition of aminopeptidase B reduced the fibrotic properties of cardiac fibroblasts, indicating a role of these aminopeptidases in fibroblast activation[35]. Furthermore, we found down-regulation of PACE4, a subtilisin-like proprotein convertase involved in the activation of bone morphogenic proteins (BMPs) and matrix metalloproteinases (MMPs)[36;37]. As BMPs have opposing effects on TGF-ß[38-41] and MMPs are involved in extracellular matrix degradation in STZ induced diabetes[42-45], the down-regulation of PACE4 may contribute to renal fibroblast activation in early diabetes.

Our list of renal genes regulated in the first week after induction of diabetes shares only a limited number of genes with genes obtained in microarray studies in more established diabetes. Down regulation of Kidney Androgen regulated protein (KAP) has been demonstrated before at later time points after induction of diabetes in several models of

murine diabetes[46]. A study performed on transgenic mice over-expressing KAP demonstrates a role for KAP in hypertension and renal alterations mediated by oxidative stress[47], although its function in diabetes remains unclear. Nevertheless, our study demonstrates that downregulation of KAP is evident in the first week after induction of diabetes. Furthermore, changes in the expression of heme-oxygenase-1, solute carrier family 7, tubulin, trefoil factor 3 and components of cytochrome P450 have been described at later time points of experimental diabetes in mice[46]. Nonetheless, less than two percent of the genes identified by us in the first week of diabetes are also identified at later time points, indicating the initiation of specific and transient gene expression profiles after induction of diabetes.
A limitation of the current study is that the employed method for analysis of the array data has limited capability to detect genes that are specifically regulated at single time points. However, as renal pre-fibrosis takes several days to develop, this limitation is not likely to affect the analysis of the genes involved in the induction of renal pre-fibrosis.

In summary, we have identified several genes associated with the induction of prefibrosis in the first week of streptozotocin induced diabetes in rats. The products of these genes were previously identified as modulators of TGF-ß signaling and included GDF15, P311, follistatin and two proprotein activating proteases. Initiation of renal prefibrosis was not associated with increased renal expression of TGF-ß mRNA expression but a transient increase in circulating latent TGF-ß may participate in the induction of renal prefibrosis. The identification of these TGF-ß modulating renal genes in early diabetes may lead to a better understanding of the pathways involved in the initiation of renal fibrosis in diabetes.

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Chapter 3

Functional effects and signaling pathways of Growth Differentiation Factor 15 in cultured human mesangial cells

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Abstract

Background

Transforming growth factor (TGF-β) is generally regarded as the key mediator in the development of renal fibrosis due to the induction of extracellular matrix (ECM) production. Growth Differentiation Factor 15 (GDF15), a secreted member of the TGF-β superfamily, is primarily expressed in the glomerulus early after induction of diabetes. In the current study, we aimed at establishing the functional implications and the signaling pathways of GDF15 on glomerular mesangial cells. Furthermore, we evaluated whether GDF15 responses resembled TGF-ß1 responses.

Methods

To investigate the signaling pathways of GDF15, human mesangial cells (THMC) were stimulated with GDF15 or TGF-β and activation of Smad pathways and MAP kinase pathway were analyzed by western blotting. The effects of preincubation with GDF15 and TGF-β on proliferation, apoptosis and α-SMA gene expression were investigated. SiRNA silencing of type 2 receptors was used to identify the receptor for GDF15 in THMC.

Results

Unlike TGF-β, GDF15 did not signal through the classical Smad 2, 3 or Smad 1, 5, 8 pathways in THMC, but signaled through the MAP kinase pathway, including p38, JNK and MEK. GDF15 had no effects on apoptosis or initiation of fibrosis in THMC but induced proliferation of THMC through activation of p38 and JNK. Silencing of type 2 receptors demonstrated that GDF15 signals through the BMPR2 receptor in THMC.

Conclusion

In conclusion, GDF15 stimulated proliferation of human mesangial cells (THMC) through activation of the MAPK members JNK and p38 and involved the BMPR2 receptor. GDF15 signaling in THMC did not employ the classical Smad pathway and was distinct from TGF-β.

Introduction

Growth Differentiation Factor 15 (GDF15), a distant member of the Transforming growth factor-β family and an early response gene, is emerging as an independent predictor of cardiovascular disease (CVD) and diabetic kidney disease. In insulin-dependent diabetes mellitus and nephropathy, GDF15 was recently identified as a predictor of all-cause and cardiovascular mortality and morbidity[1]. Furthermore, this study demonstrated that higher levels of circulating GDF15 were associated with faster deterioration of kidney function. In obese type 2 diabetic (T2DM) patients, GDF15 levels were increased compared to non-diabetic obese patients[2;3]. Furthermore, GDF15 levels correlated with insulin resistance in obese T2DM patients[4]. Interestingly, elevated levels of GDF15 have been reported before the onset of T2DM, suggesting that GDF15 may be a part of an anti-inflammatory response preceding the onset of T2DM[5]. Finally, increased GDF15 levels have been reported in diabetic pregnancies[6].

Despite the role as biomarker for progression of CVD and kidney disease, relatively little is known on the functional role of GDF15. Mice that are genetically deleted for GDF15 demonstrated no major abnormalities, except for decreased body weight, possibly through an interaction of GDF15 with adiponectin expression in fat cells[7]. Under physiological conditions, GDF15 is weakly expressed in most tissues. However, GDF15 expression is markedly increased in response to injury in various tissues[8-11], including heart and kidney[10]. As GDF15 knockout mice show increased tissue damage after cardiac injury, GDF15 is believed to be a protective factor by reducing apoptosis and influencing cellular proliferation[12-14]. Furthermore, a recent study demonstrated that GDF15 inhibits leukocyte recruitment and inflammation by inhibiting endothelial integrin activation in an experimental model of myocardial infarction[15].

In a recent experiment, renal GDF15 expression was found to be strongly upregulated in the glomeruli of diabetic rats and was associated with the induction of renal fibrosis[16]. As glomerular mesangial cells play an important role in production of glomerular extra cellular matrix (ECM) production in diabetic kidney disease, we aimed at identifying the functional effects of GDF15 in cultured human mesangial cells (THMC). Furthermore, we aimed at elucidating the signaling pathways and receptor(s) involved in GDF15 signaling in THMC.

Materials and methods

Cell Culture

Transformed human mesangial cells (THMC) and podocytes (gift from K. Sharma, UCSD, San Diego, USA) were cultured in Dulbecco's modified Eagle's medium: Nutrient mixture F-12 (DMEM/F12, 1g/L glucose; Gibco BRL) supplemented with 1.0 mg/l L-glutamine, 2% or 10% of fetal calf serum (FCS; PAA Laboratories), 100 µg/ml streptomycin (Gibco BRL) in a humidified incubator at 37 °C and 5% CO_2. When cells reached confluence, they were passaged in a 1: 20 ratio with 0.05% trypsin-EDTA (Gibco BRL); passages 6-30 were used. For incubation in high glucose and mannitol, THMC were cultured in DMEM/F12 supplemented with glucose or mannitol to 30 mM for 24 hours.

Mouse mesangial cells (MMC) cultured from transgenic mice expressing GFP under the control of a Collagen 1A promoter were grown on a collagen I (8.4 µg/cm^2) coated plates in low glucose (1 g/l) DMEM supplemented with 10% FCS and 100 µg/ml streptomycin in a humidified incubator at 37 °C and 5% CO2. MMC were seeded in 10% FCS medium in 96-well plates (Nunc) coated with collagen I. After overnight incubation, cells were treated with 50 ng/ml GDF15 and 10 ng/ml TGF-β for 48 hours. Fluorescence was measured at 509 nm.

N_2-induced hypoxia

THMC were grown in 96-well plates until 80% confluence. 24 hours prior to stimulation, the cells were placed in CO_2-independent medium, supplemented with 2% FCS. Hypoxia was induced by incubating the cells in a humidified hypoxia chamber (100% N_2) for 16 hours. Control cells were incubated at 37˚C with 5% CO_2. Subsequent to the hypoxia, cells were returned to normal growth conditions (37˚C with 5% CO_2) for 4 hours.

SiRNA transfection

SiRNA transfection was performed using Lipofectamine 2000 (Invitrogen). For this, THMC were grown in a 12 wells plate for 24 hours. SiRNA transfection was performed in duplicate, using 2 µl Lipofectamine 2000 and 80 pmol/ml siRNA in 100 µl Optimem. SiRNA's sequences used were: negative control scrambled siRNA (Ambion; AM4611), ActRIIA (Sigma), ActRIIB (Sigma), BMPRII (Sigma). After 4 hours of transfection the medium was changed for culture medium with 2% FCS for 24 hours. GDF15 (50 ng/ml) was added to the cells for 5 minutes. Cells were washed with cold phosphate-buffered saline (PBS) and subsequently lysed for protein isolation (RIPA buffer) to investigate MEK1/2 activation.

Real-Time PCR

RNA from THMC was isolated using a RNA isolation kit (NucleoSpin® RNA II, Macherey-Nagel GmbH & Co. KG). The integrity of RNA was determined using agarose gel electrophoresis, and the RNA concentration was measured spectrophotometrically at 260 nm. RNA (1 µg) was reversely transcribed into cDNA. Real-Time PCR for GDF15 was performed using AbsoluteTM QPCR SYBR® Green ROX Mix (Westburg, Leusden, Netherlands), 400 nM of primer and 2,5 µl template DNA in a total volume of 25 µl. Quantitative real-time PCR was performed at 95˚C for 15 minutes followed by 40 cycles of denaturing at 95˚C for 15 seconds and annealing/ extending at 60˚C for 1 minute. Real-Time PCR for GDF15 was performed using AbsoluteTM QPCR SYBR® Green ROX Mix (Westburg, Leusden, Netherlands), 400 nM of each primer (GDF15 F: 5'-CAGAGTTGCACTCCGAAGAC-3', GDF15 R: 5'-CACTTCTGGCGTGAGTATCC-3') and 2 µl template DNA in a total volume of 10 µl. PCR product specificity and purity were evaluated by generating a dissociation curve following the manufacturer's recommendations.

Protein isolation

Cells were seeded in 6-well plates (Nunc) at a concentration of 10.000 cells in 2 ml culture medium with 2% FCS. GDF15 (10 ng/ml, Pepro Tech, 120-28), and GDF15 (50 ng/ml) and TGF-β (10 ng/ml, Pepro Tech, 100-21) were added to the cells for 5 minutes, 30 minutes or 1 hour. Cells were washed with cold phosphate-buffered saline (PBS) and subsequently lysed in 125 µl of ice-cold RIPA buffer (1% Igepal ca-630, 1% SDS, 5 mg/ml sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM β-mercapto-ethanol, 40 µg/ml PMSF, 100 µg/ml benzamidine, 500 ng/ml pepstatin A, 500 ng/ml leupeptine and 500 ng/ml aprotinin in PBS). Protein concentrations were determined using Bio-Rad protein assay.

Western blotting

Proteins were separated by gel electrophoresis. For electrophoresis, 10 µg of total protein was combined with a 5x protein loading buffer (10% SDS, 50% glycerol, 10% β-mercapto-ethanol, 0.05% bromophenol blue in 0.33 M Tris HCL, pH 6.8) and subjected to a 4-20% Precise Protein Gel (Pierce). The separated proteins were transferred to a nitrocellulose membrane. Non-specific binding sites were blocked by incubation with 5% Skim Milk in TBS/T-20 (0.05 M Tris, 150 mM NaCl, 0.04% Tween-20) for 30 minutes. The membrane was immunoblotted with phospho-MEK antibody (sc-7383 Santa-Cruz; 1:500), anti-active phospho38 (V1211 Promega; 1:1000), phospho-Smad 1/5/8 (Chemicon Lot LV1467481;

1:500), phospho-Smad2 (Cell Signaling Ser 465/467; 1:500) and phospho-SAPK/JNK (Thr183/Tyr 185; Cell Signaling; 1:250). Primary antibodies were detected with horseradish-peroxidase-conjugated rabbit anti-mouse IgG (Dako; 1:1000) and with goat anti-rabbit IgG (Dako; 1:2000). As a housekeeping protein, GAPDH (Fitzgerald: 1:10.000) was used. After incubation, the membrane was washed with TBS/T-20 and immunoreactivity was visualized with SuperSignal West Pico Chemiluminescent kit and exposed by Gene-Gnome (Westburg B.V., Leusden, The Netherlands).

Proliferation Assay

Cells in 96-well plates (Nunc) at a concentration of 1000 cells in 100 µl culture medium were incubated with 2% FCS or 10% FCS as a control. Incubation with 50 µM MEK1 inhibitor PD98059 (Cell Signaling Technology; #9900) was performed for 1.5 hour, 50 µM p38 inhibitor SB202190 (Sigma; S7067) for 1 hour, 20 µM JNK inhibitor SP600125 (Sigma; S5567) for 1 hour. Cells were treated with GDF15 (50 ng/ml) or TGF- β (10ng/ml) for 48 hours. Proliferation was measured with the Cy-QUANT NF cell proliferation kit (Invitrogen) according to the manufacturer's instructions.

Apoptotic assay

The Apo-ONE® Homogeneous Caspase-3/7 Assay, Promega, was used to measure apoptosis following the manufacturer's description. In short, 50 µl of Apo-ONE Caspase-3/7 Reagent was added to 50 µl medium of each well of the 96-wells plate. Contents were mixed for 30 seconds and incubated for 1 hour, after which fluorescence was measured with an fluorescent plate reader.

Statistical analysis

Data are expressed as mean value ± S.E.M. Differences between groups were tested by Student's t-test and analysis of variance (ANOVA) by Microsoft Excel (2003 SP3 version). A p value less than 0.05 was considered statistically significant.

Results

GDF15 expression is upregulated by high glucose.

To investigate whether glomerular GDF15 expression may be upregulated by high glucose in diabetes, cultured human mesangial cells (THMC) and podocytes were incubated in normal tissue culture medium and in medium containing 30 mM glucose or 30 mM mannitol as an osmotic control (Figure 1). Quantitative real-time PCR demonstrated that GDF15 expression was increased almost four-fold in THMC after pretreatment with high glucose (372±150%) compared to controls (100±48%). In podocytes, the induction of GDF15 expression was less pronounced after pretreatment with high glucose (155±51%) compared to controls (100±48%). Pretreatment with mannitol did not affect GDF15 expression.

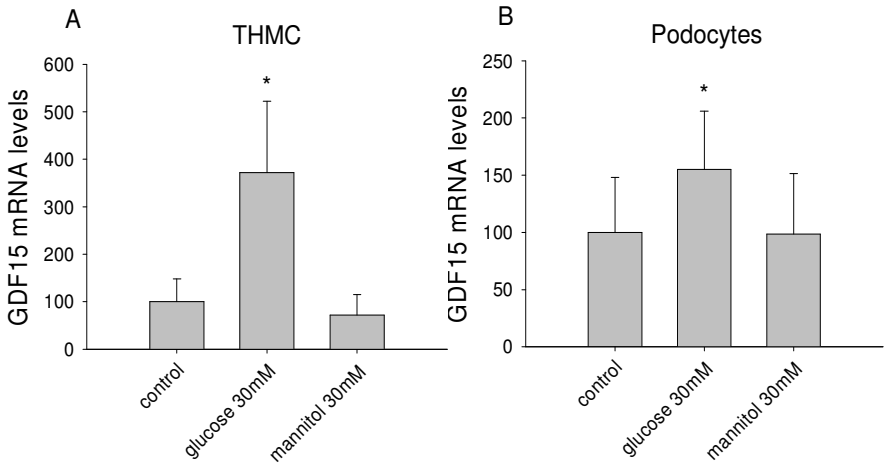

Figure 1: GDF15 mRNA expression is upregulated in cultured THMC and podocytes by high glucose. A) GDF15 expression in THMC, B) GDF15 expression in podocytes. Cell were exposed to high glucose or mannitol for 24 hours (*p< 0.05 vs control).

Effects of GDF15 on cellular proliferation, apoptosis and initiation of fibrosis in cultured THMC.

To test the functional effects of GDF15 on THMC, we investigated the role of GDF15 in cellular proliferation, apoptosis and initiation of fibrosis in cultured THMC (Figure 2). Cells were also treated with TGF-β to determine whether the effects of GDF15 were distinct from those of TGF-β.

GDF15 profoundly stimulated THMC proliferation under culture conditions with 2% FCS (185±10.9%) compared to controls (Figure 2A). The GDF15 induced proliferation of THMC was comparable to the effect of 10% FCS (189±8%). TGF-β did not significantly induce THMC proliferation (139±8.8%).

To investigate the effects of GDF15 on apoptosis of THMC, caspase activity was measured (Figure 2B). Under normal culturing conditions, pretreatment with GDF15 did not induce caspase activity while pretreatment with TGF-β caused a significant increase in caspase activity (135±5%) over controls (100±5%). As hypoxia was known to increase caspase activity in THMC (unpublished data), THMC were cultured in hypoxic conditions. Overnight exposure of THMC to hypoxia increased caspase activity (187±35%). Pretreatment with GDF15 did not affect caspase activity while TGF-β caused a further increase in caspase activity (350±16%).

To investigate the effects of GDF15 on initiation of fibrosis in THMC, the gene expression of α-smooth muscle actin (α-SMA) was determined (Figure 2C). Pretreatment with GDF15 did not affect α-SMA expression, while pretreatment with TGF-β caused increased α-SMA expression (260±33%). Furthermore, GDF15 treatment did not increase GFP expression in mesangial cells isolated from transgenic mice carrying a GFP gene under control of a collagen-I promoter (Figure 2D). In contrast, TGF-β caused increased collagen-GFP expression after 48 hours.

Together, these data demonstrate that GDF15 and TGF-β induce markedly distinct functional effects in THMC.

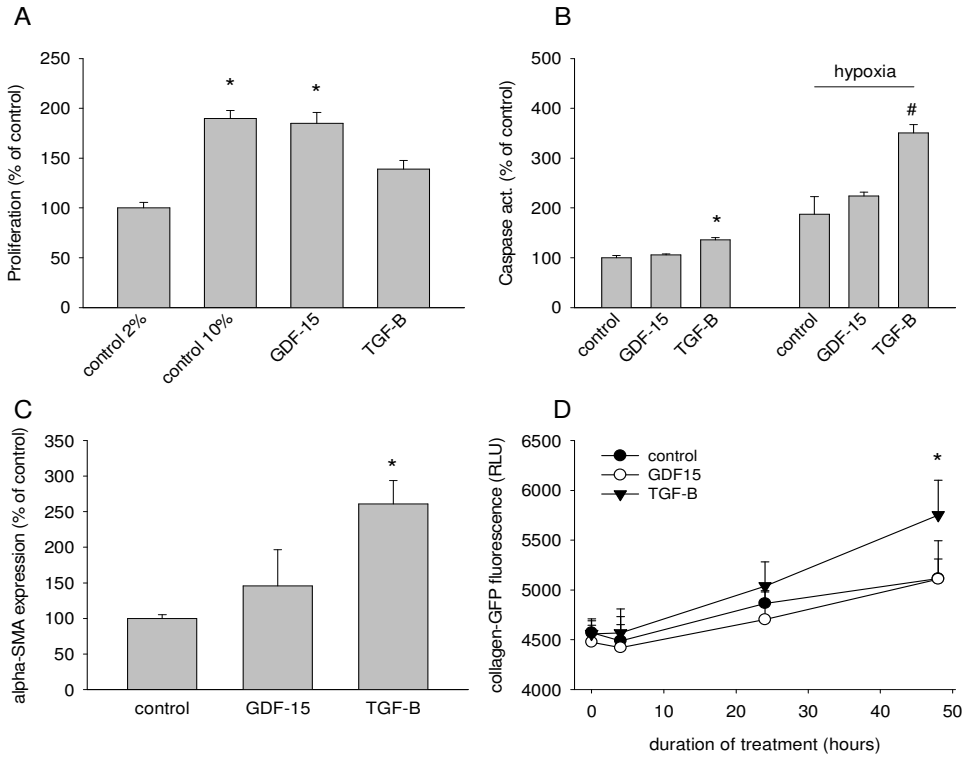

Figure 2: Effects of GDF15 and TGF-β on ***cellular proliferation, apoptosis and initiation of fibrosis in cultured THMC.*** A) GDF15 stimulates proliferation of THMC. B) GDF15 did not affect caspase activity in THMC in control and in hypoxic conditions. TGF-β increased caspase activity in both normal and hypoxic conditions. C) GDF15 did not affect α-SMA expression while TGF-β increased α-SMA expression. D) GDF15 did not alter collagen-GFP expression in mesangial cells obtained from transgenic mice with GFP under control of a collagen-I promoter. TGF-β increased collagen-GFP expression after 48 hours of incubation. (* $p<0.05$ vs control, #$p<0.05$ vs hypoxic control).

Effects of GDF15 and TGF-β on Smad and MAPK signaling in THMC

To investigate the signaling cascades activated by GDF15 and TGF-β in THMC, Western-blot was performed on cells stimulated with GDF15 and TGF-β (Figure 3).

As members of the TGF-β superfamily generally signal through Smad2/3 or Smad1/5/8 signaling, the effect of GDF15 on phosphorylation of these proteins was assessed. Using phospho-specific antibodies to Smad2/3 and Smad1/5/8, we found that GDF15 did not stimulate Smad2/3 or Smad1/5/8 in THMC at the indicated time-points (Figure 3A,B). In contrast, TGF-β activated both Smad2/3 and Smad1/5/8 after 30 minutes of stimulation.

As members of the TGF-β superfamily of signaling molecules can also signal through the noncanonical MAPK pathway, we investigated whether GDF15 activated members of the MAPK signaling cascades in THMC using phosphospecific antibodies. GDF15 transiently increased p-MEK1/2 levels after 5 minutes of stimulation (Figure 3C), Furthermore, p-JNK levels were increased after 60 minutes of GDF15 stimulation (Figure 3D) and p38 levels were increased after 30 minutes of GDF15 stimulation (Figure 3E). Stimulation with TGF-β for 30 minutes only increased p-JNK levels (Figure 3D).

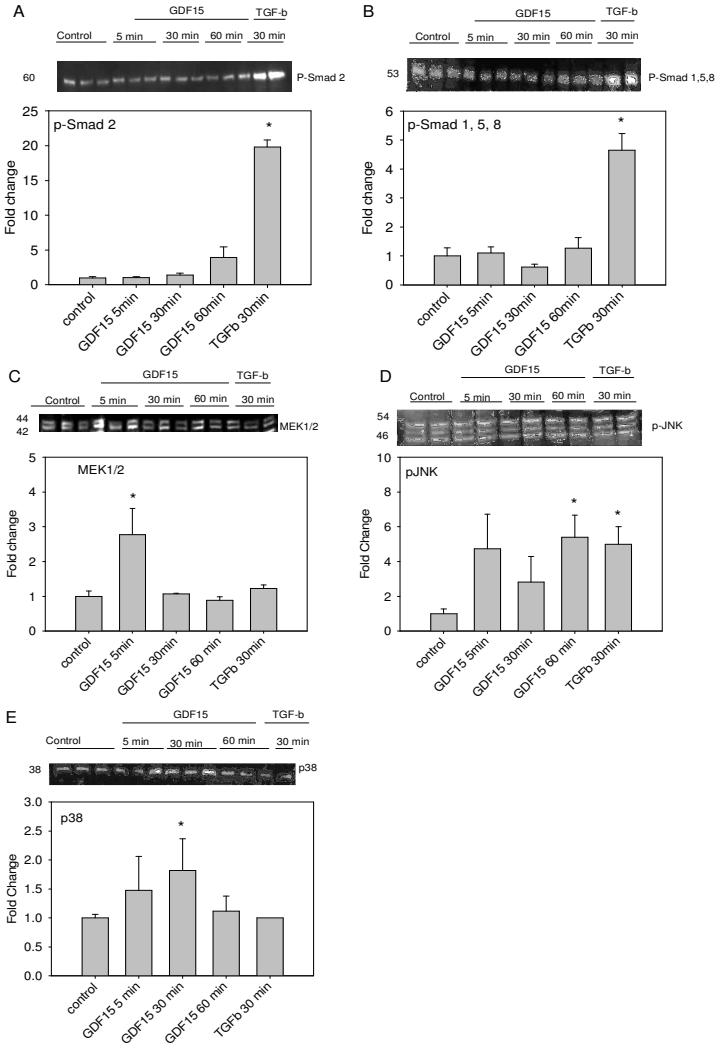

Figure 3: Effects of GDF15 and TGF-β ***on activation of Smad and MAPK signaling cascades.***

A) Exposure of THMC for 5, 30 and 60 minutes to GDF15 (50 ng/ml) did not increase p-Smad2/3. TGF-β for 30 minutes resulted in a 20 fold increase in p-Smad2/3. B) GDF15 did not increase p-Smad1,5,8 levels, while TGF-β caused a 5 fold increase in p-Smad1/5/8. C) MEK1/2 was transiently activated by GDF15 after 5 minutes. p-MEK1/2 levels were at control levels at all other time-points and after stimulation with TGF-β for 30 minutes. D) p-JNK levels were significantly increased after 60 minutes of GDF15 and 30 minutes of TGF-β stimulation. E) p-38 was significantly increased after 30 minutes of GDF15 stimulation. (*p<0.05 vs control).

Effects of MAPK inhibitors on GDF15 induced proliferation

To investigate whether GDF15 stimulates proliferation of THMC through MAPK signaling, we tested the effects of specific MAPK inhibitors on GDF15 induced THMC proliferation (Figure 5). In the absence of inhibitors (light grey bars), GDF15 increased THMC proliferation by 147±3% of controls (100±7%), confirming the effects of GDF15 on THMC proliferation. Again, a similar stimulation was achieved by incubation with 10% FCS (148±2%).

Incubation with MEK inhibitor increased basal proliferation to 130±3% (Figure 5, black bars). In the presence of MEK inhibitor, stimulation with 10%FCS and GDF15 further increased THMC proliferation to 150±2% and 143±2%, respectively. Therefore, these results demonstrate that the MEK pathway has an anti-proliferative effect on THMC. Furthermore, MEK inhibition did not inhibit GDF15 induced THMC proliferation.

Incubation with JNK inhibitor decreased basal THMC proliferation to 39±4% of control (Figure 5, white bars). Although stimulation with 10% FCS still resulted in increased THMC proliferation (74±9%), the proliferation by GDF15 was not significantly increased (50±9%).

Similarly, p38 inhibition also decreased basal THMC proliferation to 17±2% of control (Figure 5, dark grey bars). Stimulation with GDF15 still significantly increased THMC proliferation (41±5%) but this response was inhibited compared to stimulation with 10% FCS (85±16%.).

Taken together, these results demonstrate that THMC proliferation is dependent on the JNK and p38 pathway. Furthermore, JNK and p38 inhibition inhibited the effects of GDF15 on THMC proliferation.

Identification of receptors involved in GDF15 signaling in THMC

To identify the receptor(s) through which GDF15 signals in THMC, the effect of receptor silencing on MEK1/2 activation was investigated. Members of the TGF-β superfamily of signaling molecules first bind to a type 2 receptor with subsequent activation of type 1 receptors. As the type 2 receptors are the primary receptors for TGF-β superfamily ligands, we aimed at silencing the type 2A and type 2B activin receptors (ActR2A and ActR2B, respectively) and the type 2 BMP receptor (Figure 4). Transfection of THMC with SiRNA directed to ActR2A and ActR2B did not affect the response to GDF15. In contrast, transfection using a SiRNA directed to BMPR2 resulted in a complete abrogation of the effects of GDF15 on MEK1/2 activation.

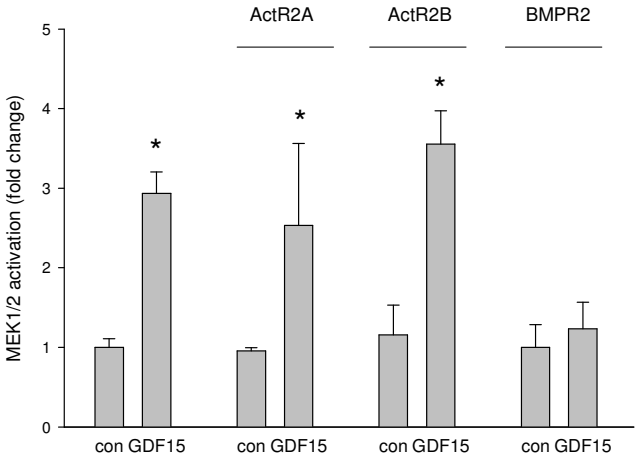

Figure 4: Effect of silencing of type 2 receptors on GDF15 mediated activation of MEK1/2. THMC were transfected with SiRNA directed to activin receptors 2A and 2B and BMP receptor 2 (* p<0.05 vs control).

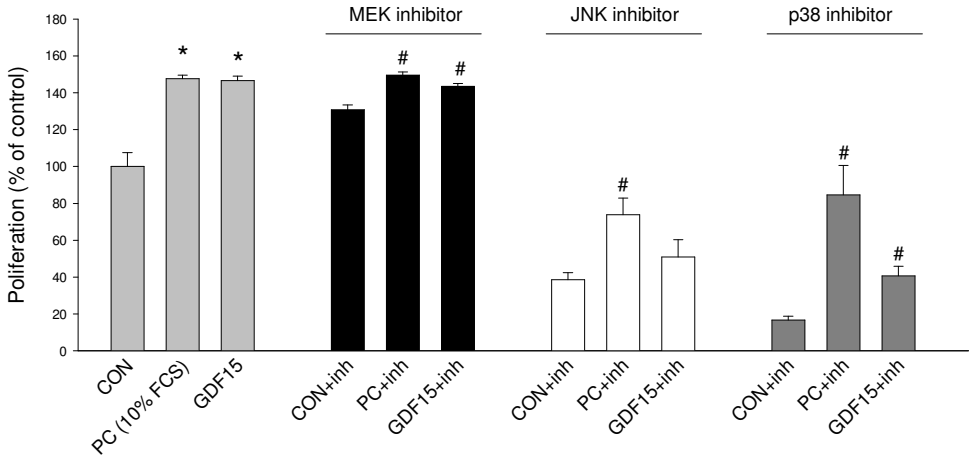

Figure 5: Effects of MAPK inhibitors on GDF15 induced proliferation of THMC. THMC were cultured in medium containing 2%FCS and treated with inhibitors for MEK, JNK and p38. Proliferation was stimulated with 10% FCS (positive control) or GDF15. (*p<0.05 vs control, #p<0.05 vs control+inhibitor).

Discussion

This is the first study to describe the signaling pathways of Growth Differentiation Factor 15 (GDF15) in cultured human mesangial cells (THMC) and identify the JNK and p38 to be prominently involved in absence of activation of the canonical Smad signaling routes. Furthermore, we are the first to identify GDF15 to signal through stimulation of the type 2 BMP receptor.

Members of the TGF-β superfamily of signaling molecules which include TGF-β, BMP, activins and GDFs generally signal through the classical (Smad) and noncanonical (MAPK) signaling pathways. Surprisingly, GDF15 did not activate the classical signaling cascade involving Smad2/3 or Smad1/5/8. This is in contrast to a previous study in which GDF15 activated the Smad2/3 signaling cascade in cultured cardiomyocytes[17;18]. Furthermore, a recent paper demonstrated that GDF15 activated the Smad1/5/8 signaling cascade in cultured cardiomyocytes but not Smad2/3[19;20]. It is unclear why GDF15 does not employ Smad signaling in THMC. However, the absence of Smad activation by GDF15 in THMC can not be explained by defects in Smad signaling in THMC, as TGF-β stimulated both Smad2/3 and Smad1/5/8 in THMC. Although the classical (Smad) and noncanonical (MAPK) signaling pathways of TGF-β, BMP and other family members have been described extensively in literature, it remains unclear whether both pathways act independently or coordinately. Some studies showed that the classical and noncanonical pathways were codependent and provided redundancy[21;22]. However, our current study in THMC demonstrates that GDF15 can activate the MAPK system without activating the Smad signaling cascades.

GDF15 did not affect fibrosis in THMC, although mesangial cells play an important role in glomerular ECM production in diabetic kidney disease (for review, see[23]). In contrast, TGF-β caused increased alpha-smooth muscle actin expression in THMC and induced collagen promoter activity in transgenic mouse mesangial cells. As Smad2/3 activation is widely recognized as a requirement for increased ECM production[24], the absence of an effect of GDF15 on Smad2/3 signaling is in line with the observation that GDF15 does not affect fibrosis in THMC.

GDF15 induced proliferation of THMC through the noncanonical MAPK signaling cascade, which was dependent on JNK and p38 signaling. This observation is in line with proliferative effects of GDF15 in several other cells types including various cancer lines,

pulmonary endothelial cells and bile duct cells[25-28]. Furthermore, several studies have reported the involvement of the MEK, JNK and p38 pathways in proliferation of cultured mesangial cells[29-31], although the relative contribution of each component varied. In our studies, inhibition of JNK and p38 led to a decrease in basal proliferation of THMC to 39% and 17% of control values, respectively. The large effect of each individual component on THMC proliferation suggests a considerable level of interaction or redundancy between JNK and p38 signaling in THMC.

Our study is the first to identify BMPR2 as the putative receptor through which GDF15 activates MAPK, as siRNA treatment downregulating the receptor abrogated GDF15 effects on MEK1/2. It has been demonstrated that BMP receptors can associate with TGF-β activated kinase TAK1 in a complex with other partners including XIAP and TAB, resulting in phosphorylation of p38 and JNK[32-34]. Furthermore, TAK1 has been shown to activate MEK1/2[35]. Together, these data suggest that GDF15 activates p38, JNK and MEK through activation of TAK1 by the BMPR2.

GDF15 did not affect basal caspase levels in THMC nor did it affect the induction of caspase activity after a noxious stimulus, indicating that GDF15 does not affect apoptosis in THMC. This is in contrast to studies performed in cardiomyocytes where GDF15 prevented cardiomyocyte apoptosis[14]. However in contrast to THMC, GDF15 increased Smad1/5/8 signaling in cardiomyocytes[36;37], a pathway known to inhibit cardiomyocyte apoptosis[38]. Therefore, the effects of GDF15 on apoptosis may only be present in cells in which GDF15 signaling is coupled to activation of Smad1/5/8.

GDF15 was found to be upregulated in the glomerulus of diabetic animals early after induction of diabetes. Nevertheless, the mechanism for GDF15 upregulation remained unclear. The incubation of cultured THMC and podocytes in conditions of high glucose increased GDF15 expression in both cell types, although induction of GDF15 expression was stronger in THMC. As osmotic controls did not change GDF15 expression, our data demonstrates that the induction of GDF15 expression in diabetes may be caused by high glucose. Nevertheless, the four-fold induction of GDF15 expression observed in THMC is less than the twenty-fold induction of renal GDF15 expression observed in whole kidney lysates of diabetic rats (Chapter 1, Figure 4). Although the results obtained in cultured THMC may not be translated directly to the *in vivo* situation, the stronger induction of

GDF15 expression *in vivo* in diabetic rats may indicate that other factors than high glucose may contribute to the induction of renal GDF15 expression in diabetes.
In conclusion, GDF15 stimulated proliferation of human mesangial cells (THMC) through activation of the MAPK members JNK and p38 and involved the BMPR2 receptor. GDF15 signaling in THMC did not employ the classical Smad pathway and was distinct from TGF-β. Consequently, GDF15 did not affect fibrosis and apoptosis in THMC. Future experiments in GDF15 knockout mice will be aimed at establishing the functional implications of GDF15 induced mesangial cell proliferation in diabetes.

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Chapter 4

Genetic deletion of Growth Differentiation Factor 15 augments renal damage in both type 1 and type 2 models of diabetes

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Abstract

Background

GDF15 is emerging as valuable biomarker in cardiovascular disease and diabetic kidney disease. Also, GDF15 represents an early response gene induced after tissue injury and studies performed in GDF15 knockout mice suggest that GDF15 plays a protective role after injury. In the current study, we investigated the role of GDF15 in the development of diabetic kidney damage in type 1 and type 2 models of diabetes.

Methods and results

Renal damage was assessed in GDF15 knockout (ko) mice and wild type (wt) mice in the streptozotocin type 1 and the db/db type 2 diabetic model. Genetic deletion of GDF15 resulted in augmented tubular and interstitial damage in both models of diabetes, despite similar diabetic states in ko and wt mice. Increased tubular damage in ko animals was associated with increased glucosuria and polyuria in both models. In both type 1 and type 2 diabetes, ko mice showed increased interstitial damage as indicated by increased α-SMA staining and collagen type 1 expression. In contrast, glomerular damage was similarly elevated in diabetic ko and wt mice. In the type 1 model, GDF15 ko mice demonstrated increased expression of inflammatory markers. In the type 2 model, elevated levels of plasma creatinine indicated impaired kidney function in ko mice.

Conclusion

These data indicate that GDF15 both protects the kidney from diabetic damage and maintains renal tubular function in both type 1 and type 2 models of diabetes.

Introduction

Growth Differentiation Factor 15 (GDF15), a distant member of the Transforming growth factor-β family and an early response gene, is emerging as an independent predictor of cardiovascular disease (CVD) in the elderly and a predictor of prognosis in patients with established CVD[1-3].

Diabetes is a common cause of CVD and a number of recent studies demonstrated an association between elevated GDF15 levels and CVD. In insulin-dependent diabetes mellitus and nephropathy, GDF15 was recently identified as a predictor of all-cause and cardiovascular mortality and morbidity[4]. The study demonstrated that higher levels of circulating GDF15 were associated with a faster deterioration of kidney function. In obese type 2 diabetic (T2DM) patients, GDF15 levels were increased compared to non-diabetic obese patients[5;6]. Furthermore, GDF15 levels correlated with insulin resistance in obese T2DM patients[5]. Interestingly, elevated levels of GDF15 have been reported before the onset of T2DM, suggesting that GDF15 may be a part of an anti-inflammatory response preceding the onset of T2DM[7]. Finally, increased GDF15 levels have been reported in diabetic pregnancies[8].

Despite the growing body of literature demonstrating that GDF15 is a valuable biomarker for (diabetic) CVD, relatively little is known on the functional role of GDF15. Mice that are genetically deleted for GDF15 demonstrate no major abnormalities, except for decreased body weight, possibly through an interaction of GDF15 with adiponectin expression in fat cells[9]. Under physiological conditions, GDF15 is weakly expressed most tissues. However, GDF15 expression is markedly increased in response to injury in various tissues[10-12], including heart and kidney[10]. As GDF15 knockout mice show increased tissue damage after cardiac injury, GDF15 is believed to be a protective factor by reducing apoptosis and influencing cellular proliferation[13-15]. Furthermore, a recent study demonstrated that GDF15 inhibits leukocyte recruitment and inflammation by inhibiting endothelial integrin activation in an experimental model of myocardial infarction[16].

In a recently performed microarray experiment, we identified GDF15 as a prominent renal factor up-regulated immediately after the induction of diabetes in rats. This and the previously reported association of GDF15 with diabetic kidney damage in patients, led us to hypothesize that GDF15 may play a role in the development of diabetic kidney damage in early diabetes. Thus, we investigated the relationship between GDF15 expression and renal damage in type 1 diabetic rats. In addition, the role of GDF15 in the development of renal diabetic damage was assessed in wild type and GDF15 knockout mice models with type 1 and 2 diabetes.

Materials and methods

Animal models

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Groningen, the Netherlands).

Streptozotocin induction of type 1 diabetes in rats

Male Wistar rats (weight 250 – 270 g, 7-8 weeks old, Harlan) were housed under standard conditions with free access to food (standard rat chow) and drinking water throughout the study. Diabetes was induced in rats by a single injection of streptozotocin (STZ) in citrate buffer (60mg/kg i.v). A sham group injected with citrate buffer only (n=10) was included. STZ treated rats were sacrificed at day 1 (n=6), 2 (n=7), 4 (n=5) and 7 (n=6) after induction of diabetes. At sacrification, rats were anesthetized with isoflurane (2%) and an aortic blood sample was collected. Plasma was isolated and stored at -80˚C. The kidneys were perfused with saline and removed. Kidney sections were frozen in liquid nitrogen, stored at -80˚C and used for RNA isolation. For GDF15 immunohistochemistry, kidney sections were embedded in tissue-tek OCT and frozen in liquid nitrogen.

Streptozotocin induction of type 1 diabetes in mice

For measurement of renal GDF15 expression in the first week of diabetes, male mice (8-10 weeks old; C57BL/6) were made diabetic by repeated i.p. injections of a low dose streptozotocin (35mg/kg STZ) during five consecutive days. STZ was prepared fresh daily and dissolved in 0.1 M citrate buffer (pH 4.38). Mice were sacrificed on day 0 (n=5), 1 (n=5), 2 (n=5), 4 (n=5), 7 (n=4) after the last STZ injection. A control non-diabetic group received saline i.p. and was sacrificed on day 0 (n=2). Blood samples and both kidneys were collected at the end of the experiment for analysis of glucose levels (blood) and GDF15 expression (kidneys).

Streptozotocin induction of type 1 diabetes in GDF15 wild type and knockout mice

Male wild-type mice and GDF15 knockout mice (8-10 weeks old) were bred from 5 breeding pairs generously provided from the Johns Hopkins University Baltimore, Maryland, USA. All mice were housed under standard conditions with free access to food and drinking water throughout the study. Wild-type and knockout mice were made diabetic by low dose STZ injections as described above (50mg/kg i.p, 5-6 mice per group). Healthy wild-type and knockout groups consisted of 7 mice. Urine samples were collected to measure basal proteinuria, albuminuria and urine creatinine. Mice were placed in metabolic

cages for 24 hours after last day of injection (day 0), week 1 and week 2. Mice were anesthetized with isoflurane (2%) and a blood sample was collected. Glucose measurement was performed from fresh blood immediately after sacrification with an Accu-chek® glucose meter (Roche Diagnostics, Netherlands). Plasma was collected and stored at -80˚C. Kidneys were perfused with saline and removed. Midcoronal slices from kidney were fixed in 4 % paraformaldehyde, processed for embedding and further used for immunohistochemistry. For protein and RNA isolation, tissues were snap frozen in liquid nitrogen, and stored at -80˚C until further processing.

Type 2 diabetes in mice

Five female heterozygous db/+ mice B6.Cg-Dock7m +/+ Lepr db/J mice (7 weeks of age) were purchased from The Jackson Laboratories. To evaluate the effect of genetic deletion of GDF15 in the db/db type 2 diabetic mouse model, homozygous GDF15 knockout mice were interbred with heterozygous db/+ mice. The desired male genotypes db/db GDF15+/+ (n=10) and db/db GDF15-/- (n=8) were subsequently obtained by successive rounds of inbreeding. Non-diabetic db/+ GDF15-/- mice (n=6) were used a controls. Genotyping for mouse leptin receptor mutation was performed as described previously[17]. Mice were housed under standard conditions with free access to food and drinking water throughout the study. Body weight and blood pressure were measured weekly. Mice were placed in metabolic cages for 24 hours when aged 6, 8, 10, 12, 14, 16 and 18 weeks, and food and water intake were measured. Urine samples were collected to measure basal proteinuria, creatinine, glucose, and concentration of sodium and potassium. Blood samples were obtained from the tail vein or eye socket. Plasma glucose concentrations were determined by the Accu-chek® glucose meter (Roche Diagnostics, Netherlands), HbA1c levels were analyzed by hematology analyzer (ADVIA120, SIEMENS). At an age of 18 weeks mice were anesthetized with isoflurane (2%) and blood samples were collected. Fresh blood was used for HbA1c, LDL, HDL, cholesterol and creatinine measurements (Demecal Set, LabAnywhere, Haarlem, Netherland). Plasma was isolated and stored at -80˚C. The kidneys were frozen in liquid nitrogen, stored at -80˚C and used for RNA isolation.

Real-Time PCR

RNA from kidneys was isolated using a RNA isolation kit (NucleoSpin® RNA II, Macherey-Nagel GmbH & Co. KG). The integrity of RNA was determined using agarose gel electrophoresis, and the RNA concentration was measured spectrophotometrically at 260 nm. RNA (1 µg) was reversely transcribed into cDNA. Real-Time PCR for GDF15 was

performed using Absolute™ QPCR SYBR® Green ROX Mix (Westburg, Leusden, Netherlands), 400 nM of primer and 2,5 µl template DNA in a total volume of 25 µl. Quantitative real-time PCR was performed at 95˚C for 15 minutes followed by 40 cycles of denaturing at 95˚C for 15 seconds and annealing/ extending at 60˚C for 1 minute. Real-Time PCR for KIM-1, Collagen1, Nephrin, Podocin, GapdH[18], MIP-2, KC, ICAM[19], and glucose transporters[20] was performed using Absolute™ QPCR SYBR® Green ROX Mix (Westburg, Leusden, Netherlands), 400 nM of each primer (table 1) and 2 µl template DNA in a total volume of 10 µl. Quantitative real-time PCR was performed at 95˚C for 15 minutes followed by 40 cycles of denaturing at 95˚C for 15 seconds and annealing/ extending at 60˚C for 30 seconds, 75˚C for 30 seconds. PCR product specificity and purity were evaluated by generating a dissociation curve following the manufacturer's recommendations.

Table 1: Sequences of PCR primers

gene	Forward primer (5'→3')	Reverse primer (5'→3')
GDF15	ACCTGCTGAGCCGACTGCAT	CCAATCGCACCTCTGGACTG
KIM-1	AAATCCAGAGATTCCCACACG	GTCGTGGGTCTTCCTGTAGC
Collagen 1	GGCCATTGGTGGTGCTGAC	TAAAGGAGGAAACGGCAAAG
Nephrin	TCTTCAAATGCACAGCCACCA	CAAAGCCAGGTTTCCACTCCA
Podocin	AAGGACAGATATGGGCACTGTCA	CCAGGAGCACCTAAGCTATGGAA
GapdH	AAATGGTGAAGGTCGGTGTG	TGAAGGGGTCGTTGATGG
MIP-2	CCAAGGGTTGACTTCAAGAAC	AGCGAGGCACATCAGGTACG
KC	CAATGAGCTGCGCTGTCAGTG	CTTGGGGACACCTTTTAGCATC
ICAM	TGTTTCCTGCCTCTGAAGC	CTTCGTTTGTGATCCTCCG
GLUT1	GGTGTTTGGCTTAGACTCCATC	GCTCTACAACAAACAGCGACAC
GLUT2	GAAGAGGAGACTGAAGGATCTGC	GTAGCAGACACTGCAGAAGAGC
SGLT1	GGAAGCATATGACCTGTTCTG	GACAGAGTGACATCAGACAACAC
SGLT2	CTTCTGTCGGGTACACTACCTC	CCTGGTTGTAGCTCATGACATC

Albumin levels in urine

Albumin urine levels from 24h urine collections were measured using an ELISA kit for mouse Albumin (Bethyl Laboratories, Montgomery, TX), according to the manufacturer's instructions.

Glucose, creatinine levels and proteinuria in urine

Urine from 24h collections were stored at -20 °C until analyses were performed. Urinary glucose, creatinine and protein were measured by multi-test analyser system (Roche

Modular, Mannheim, Germany). Urinary glucose, UAE, Albumin/Creatinine and proteinuria were calculated from 24h urinary volume.

Immunohistochemistry

To evaluate GDF15 localization in the diabetic rat and mouse kidney, a staining for GDF15 was performed on kidneys collected at day 1 after the STZ mediated induction of diabetes. Frozen sections (4 µm) were cold-dried and fixed in acetone. Sections were incubated with the primary antibody GDF15 (C-12): sc-46248 (dilution 1:20), followed by incubation with FITZ-labelled secondary antibody (Southern Biotechnology Associates, Birmingham, Alabama, USA).

Kidneys fixed in paraformaldehyde were used for α-SMA staining. Four µm sections were cut, deparaffinized, hydrated, processed with 1mmol/EDTA (pH 9.0) for antigen retrieval. All steps were according to the Vector MOM kit protocol. To evaluate pre-fibrosis after diabetic injury, sections were stained for α-smooth muscle actin (α-SMA, mouse monoclonal anti-α-smooth muscle actin, Sigma Chemical Co, St Louis, MO, USA) in 1:100 dilution, with MOM diluent certifying negative staining. Peroxidase activity was developed by incubation with AEC (Dako) The expression of α-SMA was measured using computer-assisted morphometry. Total staining was evaluated at a magnification of 20x. Glomeruli and arteries were excluded from measurements. α-SMA staining was divided by the area measured and expressed as a percentage. An average score was calculated per section.

To evaluate the renal damage after diabetic injury, sections were stained for kidney injury molecule (KIM-1), a marker of tubular damage (rabbit polyclonal, generously provided by Dr. H. van Goor, UMCG). Paraffin sections were dewaxed and subjected to antigen retrieval in 0.1M Tris/HCl buffer, pH9, by overnight incubation at 80^0C. A 2-step immunoperoxidase technique was used, as previously described[21]. Control slides, in which the primary antibody was replaced with PBS were consistently negative. The expression of KIM-1 was measured using computer-assisted morphometry. Total staining was evaluated at a magnification of 20x and expressed as a percentage. Evaluation of the stainings and morphometric analysis were performed in a blinded manner.

Morphological analysis

Four µm thick formalin fixed sections were deparaffinized and stained for periodic acid Schiff (PAS) for quantification of focal glomerulosclerosis (FGS) and tubular injury. FGS was semi quantitatively scored in a blinded fashion by determining the level of mesangial

expansion and focal adhesion in each quadrant in a glomerulus and expressed on a scale from 0 to 4. If 25% of the glomerulus was affected, it was scored as 1, 50% as 2, 75% as 3 and 100% as 4. In total, 50 glomeruli per kidney were analyzed, and the total FGS score was calculated by multiplying the score by the percentage of glomeruli with the same FGS-score. Thus, the total FGS score ranged from 0 to 200. Histological changes of tubular morphology were evaluated by assessment of four markers of damage: tubular necrosis, loss of brush border, denudation of basement membrane and intraluminal casts. Each parameter was graded on a scale from 0 to 3, according to the extent of the injury (0: <5%; 1: 5-25%; 2: 25-75%; 3: >75%). In total, 30 tubules per kidney were analyzed, and the histological score was calculated. Thus total histological score ranged from 0 to 90.

Cell culture

Transformed human mesangial cells (THMC) were cultured in Dulbecco's modified Eagle's medium: Nutrient mixture F-12 (DMEM/F12; Gibco BRL) supplemented with 1,0 mg/l L-glutamine, 2% or 10% of fetal calf serum (FCS; PAA Laboratories), 100 µg/ml streptomycin (Gibco BRL) in a humidified incubator at 37 °C and 5% CO2. For proliferation experiments, cells were plated in a 96-well plate at a concentration of 1000 cells in 100 µl culture medium with 2% FCS. As a positive control for cell growth, cells were seeded in culture medium with 10% FCS. To investigate the effect of GDF15 on proliferation, cells were treated with 50 ng/ml GDF15 for 48 hours. In addition, cells were treated with TGF-β (10 ng/ml) or a combined treatment of TGF-β and GDF-15. The CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen) was used to measure cell proliferation. Medium was removed and 100 µl of 1x dye binding solution was added to each well. Cells were incubated for 30 minutes at 37˚C with 5% CO_2. Fluorescence was measured using a fluorescent plate reader with excitation at 485 nm and emission detection at 530 nm.

Mink lung epithelial cells (MLEC) were cultured in low glucose (1 g/l) DMEM supplemented with 10% FCS and 100 µg/ml streptomycin in a humidified incubator. Cells were seeded in 6-well plates (Nunc) at a concentration of 10.000 cells in 2 ml culture medium with 2% FCS (THMC), 10% FCS (MLEC). After overnight incubation, cells were treated with 50 ng/ml GDF15 and 3 ng/ml TGF-β) for 5 minutes. Unstimulated cells in 2% FCS or 10% FCS were taken as a control.

Mouse mesangial cells (MMC) cultured from transgenic mice expressing GFP under the control of a Collagen 1A promoter were grown on a collagen I (8.4 µg/cm^2) coated plates in low glucose (1 g/l) DMEM supplemented with 10% FCS and 100 µg/ml streptomycin in a

humidified incubator at 37 °C and 5% CO2. MMC were seeded in 10% FCS medium in 96-well plates (Nunc) coated with collagen I. After overnight incubation, cells were treated with 50 ng/ml GDF15 and 10 ng/ml TGF-β for 48 hours. Fluorescence was measured at 509 nm.

Protein isolation and Western blotting

Cells (6-well dish format) were washed with cold phosphate-buffered saline (PBS) and subsequently lysed in 125 µl of ice-cold RIPA buffer (1% Igepal ca-630, 1% SDS, 5 mg/ml sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM β-mercapto-ethanol, 40 µg/ml PMSF, 100 µg/ml benzamidine, 500 ng/ml pepstatin A, 500 ng/ml leupeptine and 500 ng/ml aprotin in PBS). Protein concentrations were determined using Bio-Rad protein assay. Proteins were separated by gel electrophoresis. For electrophoresis, 10 µg of total protein was combined with a 5x protein loading buffer (10% SDS, 50% glycerol, 10% β-mercapto-ethanol, 0.05% bromophenol blue in 0.33 M Tris HCL, pH 6.8) and subjected to a 4-20% Precise Protein Gel (Pierce). The separated proteins were transferred to a nitrocellulose membrane. Non-specific binding sites were blocked by incubation with 5% Skim Milk in TBS/T-20 (0.05 M Tris, 150 mM NaCl, 0.04% Tween-20) for 30 minutes. The membrane was immunoblotted with phospho-Smad2 (Cell Signaling Ser 465/467; 1:500), phospho-Smad 1/5/8 (Chemicon Lot LV1467481; 1:500), or GAPDH antibody (Fitzgerald industries 10R-6109a, 1:10.000). Primary antibodies were detected with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Dako; 1:2000) or with rabbit anti-mouse IgG (Dako; 1:1000). After incubation, the membrane was washed with TBS/T-20 and immunoreactivity was visualized with SuperSignal West Pico Chemiluminescent kit and exposed by Gene-Gnome (Westburg B.V., The Netherlands).

Statistical analysis

Data are expressed as mean value ± S.E.M. Differences between groups were tested by Student's t-test and analysis of variance (ANOVA) by Microsoft Excel (2003 SP3 version). A p value less than 0.05 was considered statistically significant.

Results

Renal GDF15 expression is up-regulated early after STZ induced diabetes in both rats and mice.

In a microarray experiment aimed at identifying renal factors associated with the induction of renal damage in early diabetes in rats, we identified GDF15 as one of the most prominent factors up-regulated within the first week of diabetes (Mazagova et al., submitted). To substantiate the up-regulation of GDF15, type 1 diabetes was induced by standard protocols and renal expression of GDF15 was investigated using RT-PCR and immunostaining. Real-time PCR demonstrated a very strong up-regulation of GDF15 in the first week of STZ induced diabetes in rats (Figure 1A). In addition, real-time PCR on kidneys obtained from diabetic mice showed that GDF15 was also substantially up-regulated in the low-dose STZ model in mice (Figure 1B). Immunohistochemistry demonstrated that GDF15 staining was primarily present in the glomeruli in both rats and mice (Figure 1C and 1D, respectively). Thus, these data demonstrate an increased glomerular expression of GDF15 early after induction of type 1 diabetes in both rat and mouse.

Metabolic parameters of type 1 diabetic wt and GDF15 ko mice.

To investigate the functional implications of GDF15 in type 1 diabetic kidney disease, we compared the induction of renal diabetic damage in wild-type and GDF15 knockout mice in the first two weeks after induction of diabetes by STZ. The principal animal characteristics are shown in Table 2. In non-diabetic mice, body weight of GDF15 ko mice was significantly lower than in wt mice. Further, non-diabetic GDF15 ko mice displayed increased kidney weight and kidney to body weight ratio compared to non-diabetic wt mice. Food and water intake, urine output and urine glucose levels were similar in non-diabetic GDF15 ko and wt mice.

Induction of diabetes resulted in elevated blood glucose levels at day 7 and day 14, which did not differ between GDF15 ko and wt mice (Table 2). Body weights decreased after induction of diabetes in wt and GDF15 ko mice at both time points. Food intake was similar for diabetic wt mice compared to nondiabetic controls. In contrast, food intake was increased in diabetic GDF15 ko at both time points compared to nondiabetic ko mice. Water intake and urine output in diabetic wt animals were increased by day 7, but returned to normal levels at day 14. In contrast, water intake and urine output in diabetic GDF15 ko mice were significantly increased at day 7 and 14 compared to nondiabetic wt and ko mice. Urinary glucose excretion was increased in all diabetic mice but was significantly higher for diabetic GDF15 ko mice compared to diabetic wt mice.

Induction of diabetes resulted in a similar increase in urinary albumin excretion (UAE) and albumin/creatinine ratio in both wt and GDF15 ko mice. Despite increased UAE, animals did not show proteinuria.

These data demonstrated that genetic deletion of GDF15 did not change the diabetic state, but resulted in more severe symptoms of diabetes.

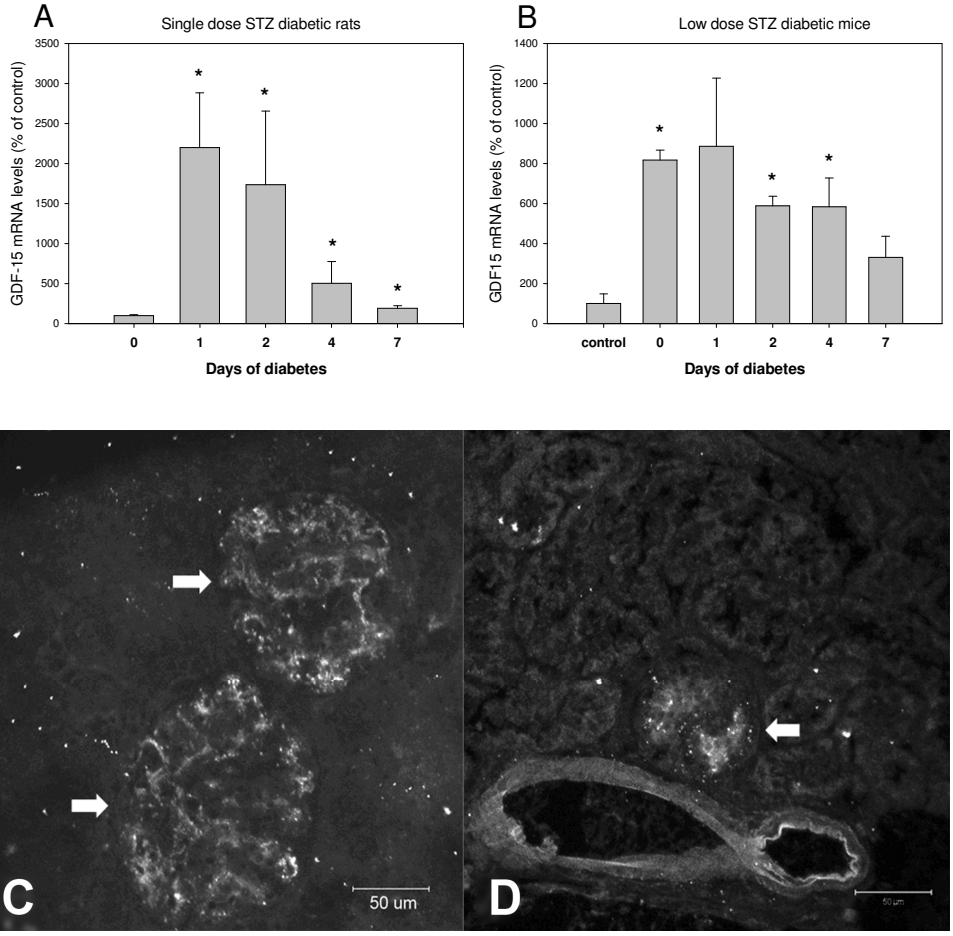

Figure 1: Renal GDF15 expression and immunostaining in diabetic rats and mice. A) Real-time PCR for renal GDF15 expression in diabetic rats after a single dose of STZ. * $p < 0.05$ control vs diabetic animals. B) Renal GDF15 expression in the low dose STZ model in mice. C,D) GDF15 immunohistochemistry at day 1 of diabetes demonstrating GDF15 staining in the glomeruli of rats (C) and mice (D), respectively (bars are 50 micrometer).

Table 2: Animal characteristics of type 1 diabetic mice.

	control		diabetic			
Parameter	**wt control n=7**	**ko control n=7**	**wt day 7 n=6**	**ko day 7 n=5**	**wt day 14 n=6**	**ko day 14 n=6**
BW beginning (g)	26.7±0.7	23.7±0.3*	26.7±0.8	25.1±0.9	24.3±1.3	22.9±0.5
BW end (g)	26.6±0.4	24.6±0.2*	24.6±0.6	22.5±1	20.6±1.1#	21.7±0.9
Blood glucose (mmol/l)	10.8±1.4	12.7±0.6	19.4±2.5#	21.4±2.7#	19.9±1.3#	20.5±3.2#
L kidney (mg)	178±5	196±8	179±6	203±7*	148±11	173±13
R kidney (mg)	189±6	207±8	190±8	202±7	162±11	202±8*
Liver (mg)	1415±50	1209±52*	1466±41	1326±105	1096±70	1327±137
Heart (mg)	150±7	145±6	136±7	133±3	120±6	138±8
L kidney/BW (mg/g)	6.7±0.1	8.0±0.3*	7.3±0.1#	9.1±0.3*#	7.3±0.5	8.1±0.7
R kidney/BW (mg/g)	7.1±0.2	8.4±0.3	7.7±0.2	9.0±0.3*	7.9±0.4	9.3±0.2*#
liver/BW (mg/g)	53.3±1.9	49±2.0	59.7±1.1#	58.8±2.5*#	53.3±1.9	60.5±4.6*#
heart/BW (mg/g)	5.7±0.2	5.9±0.2	5.5±0.1	6.0±0.2	5.9±0.3	6.4±0.4
Food intake (g/day)	2.9±0.1	2.4±0.4	3.2±0.4	4.0±0.5#	1.8±0.5	4.2±1.1#
Water intake (ml/day)	4.1±0.2	4.3±0.2	5.6±0.6	11.2±1.9*	3.8±0.6	11.9±3.6*
Urine output (ml/day)	2.1±0.3	1.6±0.1	3.3±0.6	8.1±1.7*#	1.9±0.8	8.1±3.2*#
Urine glucose (mg/day)	1.9±0.4	0.7±0.2	100±38#	278±107*#	97.7±93.1	606±297*#
UAE (ug/day)	102±17	98.3±31.0	653±140#	461±59#	658±360#	484±146#
Alb/Creat ratio (ug/mg)	111±17	82±21	1128±247#	1161±287#	863±36#	779±126#
Proteinuria (mg/day)	3.3±0.6	2.7±0.4	3.0±0.5	3.0±0.7	1.1±0.3	3.9±1
HbA1c (%)	4.0±0.1	4.1±0.5	4.0±0.1	3.9±0.2	4.8±0.3	4.0±0.2

Data are given as mean ± SEM, n= number of animals; BW: body weight, L: left, R: right, UAE: urine albumine excretion, Alb/Creat ratio: urine albumin to creatinine ratio; HbA1c: glycated hemoglobin (* $P < 0.05$ GDF15 ko versus wt, # $P < 0.05$ diabetic versus control mice)

Interstitial and tubular damage in type 1 diabetic wt and GDF15 ko mice.

The expression of interstitial α-smooth muscle actin (α-SMA), a marker of renal pre-fibrosis, was determined using immunohistochemistry and morphometric analysis. In non-diabetic wt and ko mice, α-SMA expression was predominately expressed in arteries

with marginal expression in the tubular interstitium (Figure 2A and 2D, respectively). Interstitial α-SMA was more apparent after one and two weeks of diabetes both in wt and GDF15 ko mice (panel 2B,C,E,F, respectively). Quantification of α-SMA staining demonstrated an approximately doubling of interstitial α-SMA expression after one week of diabetes in wt mice which remained at a similar level in week 2 (Figure 3A). GDF15 ko mice showed a similar increase in α-SMA staining at week 1, followed by a substantial further increase at week 2.

Tubular damage was assessed by immunonhistochemical staining for Kidney Injury Marker 1 (KIM-1). KIM-1 staining was confined to tubules and was detected in kidneys of diabetic GDF15 ko and diabetic wt mice at both time points investigated (Figure 2H,I,K,L). Morphometric quantification demonstrated increased KIM-1 expression after one and two weeks of diabetes in both GDF15 ko and wt mice (Figure 3B) with no apparent differences between groups.

Renal damage markers in type 1 diabetic wt and GDF15 ko mice.

To further assess renal damage, we quantified the mRNA expression of renal damage markers by real-time PCR on RNA isolated from kidney sections containing medulla and cortex. Induction of diabetes was associated with a more pronounced increase in KIM-1 mRNA expression in the GDF15 ko group over wt at week 1 (Figure 4A). At week 2, KIM-1 expression in wt mice returned to control levels but KIM-1 expression in GDF15 was still significantly elevated in GDF15 ko mice, although significantly lower than at week 1. Renal expression of type 1 collagen mRNA expression (Figure 4B) showed a similar pattern as KIM-1. Type 1 collagen mRNA expression was increased in wt mice at week 1 and normalized by week 2. In GDF15 ko, type 1 collagen mRNA expression at week 1 was significantly higher than in wt mice and remained elevated over controls by week 2, although at a lower level.

To assess injury to podocytes, we measured the renal expression of nephrin and podocin. There was no significant difference in the expression of these podocyte markers between diabetic GDF15 ko and wt mice, neither between diabetic groups and control groups (Figure 4C, D).

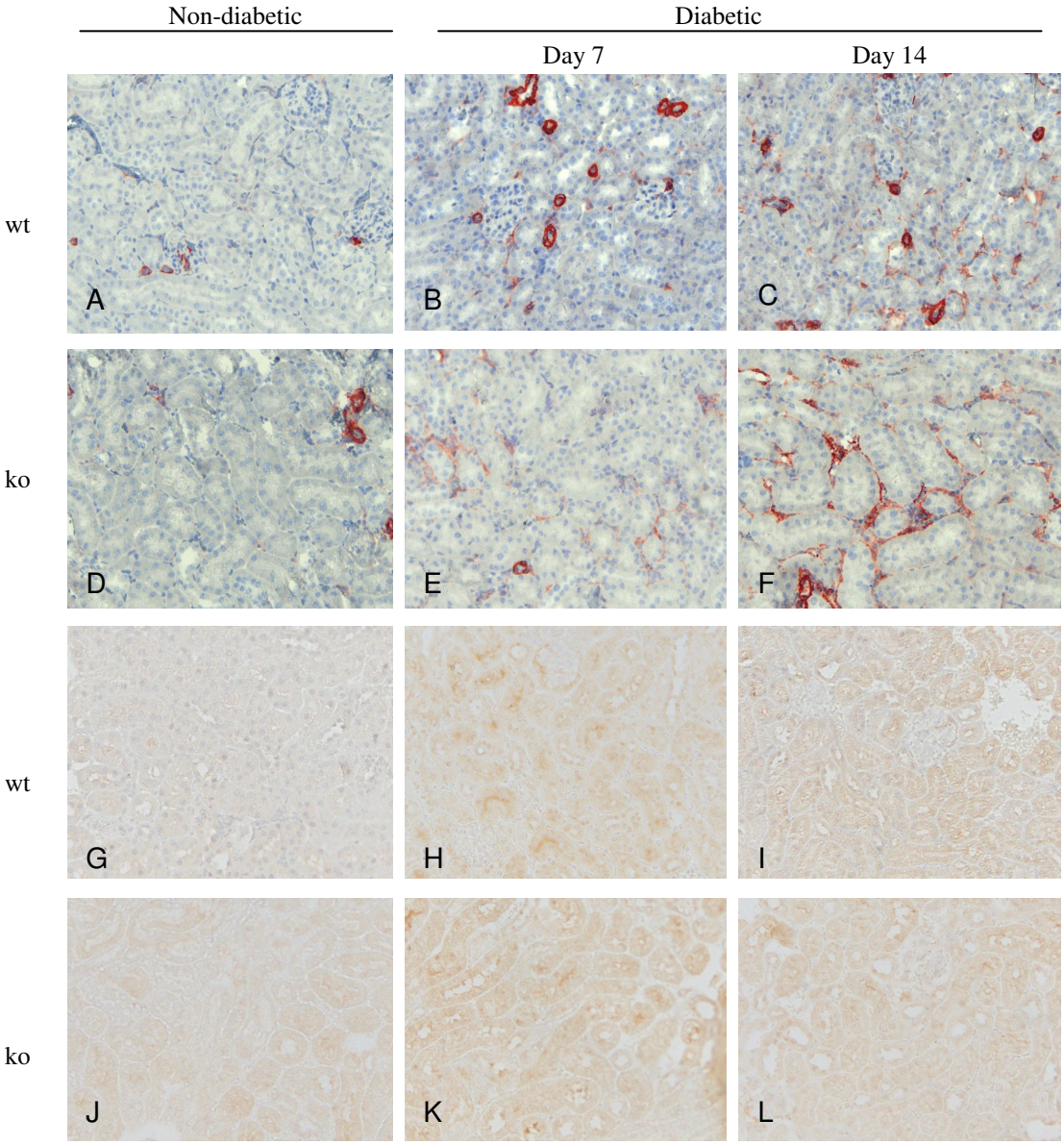

Figure 2: Representative pictures from immunohistology, showing (A-F) interstitial α-SMA expression, (G-L) KIM-1 expression in tubular epithelial cells. Pictures from control mice are shown in the first panel.

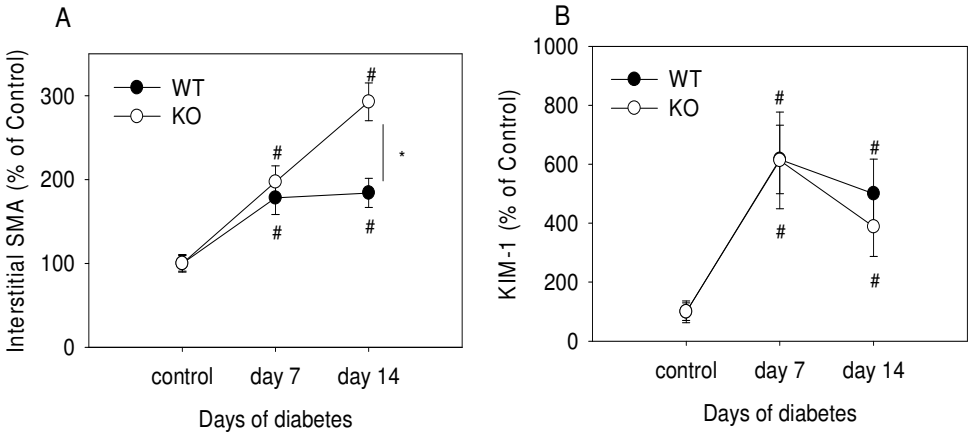

Figure 3: Interstitial pre-fibrosis (α-SMA) and tubular damage (KIM-1). (A) Morphometrical analysis of α-SMA staining in diabetic kidneys showed significantly more α-SMA expression in the GDF15 knockout mice compared to wildtype mice. (B) Increased KIM-1 expression was found in the diabetic mice (*$p < 0.05$ GDF15 ko vs wt, # $p< 0.05$ diabetic vs control mice)

Expression of inflammatory markers in type 1 diabetic wt and GDF15 ko mice.

To assess early renal inflammation after induction of diabetes, we measured the expression of chemokine KC, intracellular adhesion molecule ICAM-1 and macrophage inflammatory protein MIP-2 (Figure 4E, F, G). At day 7 of diabetes, the expression of KC, ICAM-1 and MIP-2 were all significantly increased in GDF15 ko mice compared to non-diabetic GDF15 ko mice. The expression of these inflammatory markers at day 7 of diabetes was less pronounced in wt mice and did not reach significant levels for KC and MIP-2. At day 14 of diabetes, the expression of KC, ICAM-1 and MIP-2 did not differ between GDF15 ko and wt mice.

Expression of renal glucose transporters in type 1 diabetic wt and GDF15 ko mice.

As diabetic GDF15 ko mice demonstrated increased urinary glucose excretion and increased urinary volume, we examined the renal expression of glucose transporters (Figure 4H, I, J, K). We observed considerable variation in renal glucose transporter expression, especially at day 7 of diabetes. However, the expression of Glut1, Glut2, SGLT1 and SGLT2 did not significantly differ between GDF15 ko and wt mice at any of the time points investigated.

In summary, our results in a model of type 1 diabetes indicated more renal damage in diabetic GDF15 ko mice over diabetic wt mice, despite similar blood glucose levels. Diabetic GDF15 ko mice showed increased urinary glucose loss, increased urine production, increased α-SMA staining, increased type 1 collagen mRNA expression, increased KIM-1 mRNA expression and increased expression of inflammatory markers KC, ICAM1 and MIP-2 over diabetic wt controls.

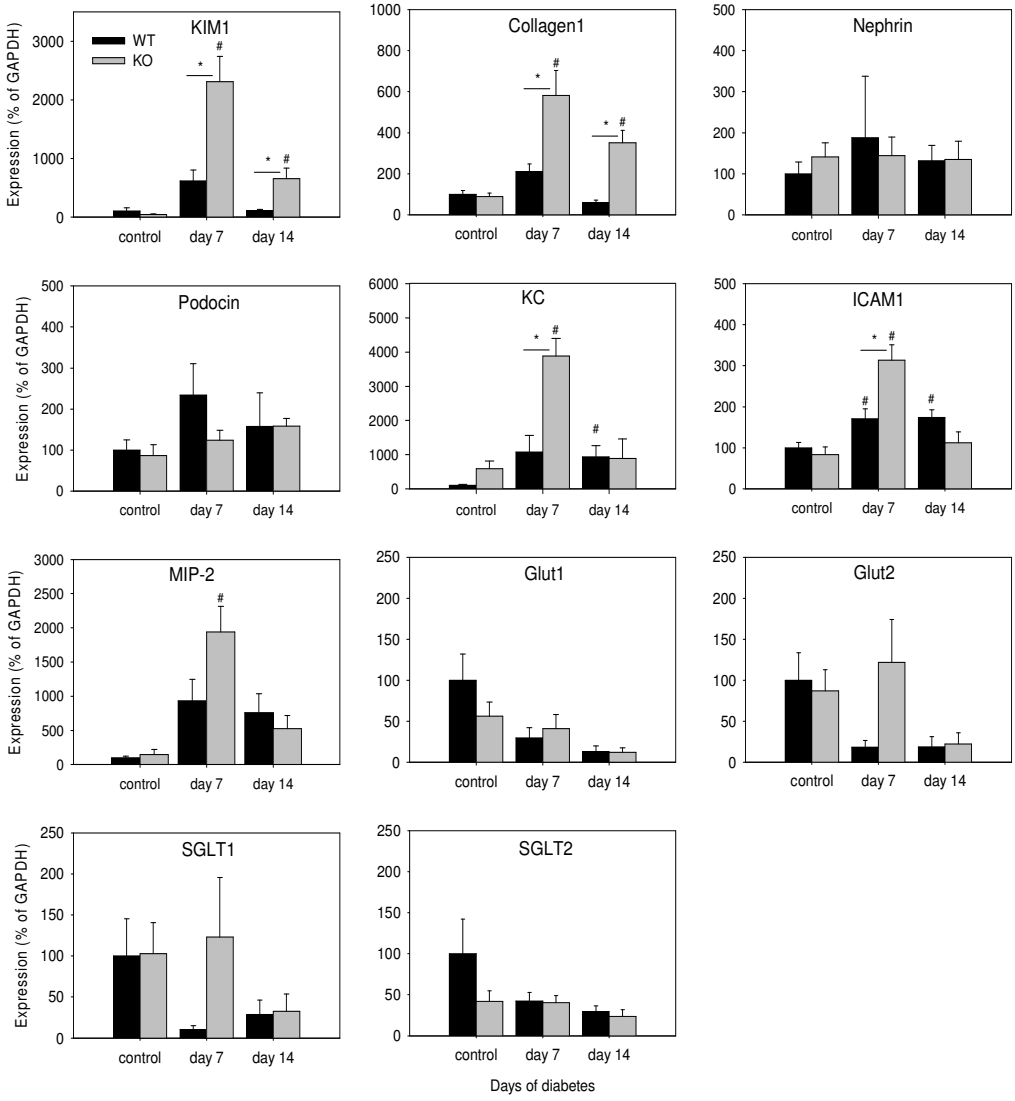

Figure 4: Expression of renal markers in wild type and GDF15 knockout mice after STZ mediated induction of diabetes (* p<0.05 GDF15 ko vs wt, # p<0.05 diabetic vs non-diabetic control mice)

Genetic deletion of GDF15 in the type 2 diabetic db/db model.

To substantiate the effect of genetic deletion of GDF15 in accelerating renal damage in a type 2 model of diabetes, we interbred GDF15 ko mice with db/+ mice. The progression of diabetic nephropathy was monitored in male db/db GDF15-/- (diabetic GDF15 ko), db/db GDF15+/+ (diabetic wt) and in db/+ GDF15-/- control mice (non-diabetic GDF15 ko) from an age of 6 weeks onwards.

Body weights (Figure 5A) were similar in both diabetic GDF15 ko and diabetic wt mice and were significantly increased over non-diabetic GDF15 ko controls. Food intake (Figure 5B) in diabetic wt and diabetic GDF15 ko mice was increased over non-diabetic GDF15 ko controls throughout the experiment. Significant levels of proteinuria did not develop in any of the diabetic groups (Figure 5F). Blood pressure was similar in all groups (Figure 5I).

Water intake (Figure 5C) and urine output (Figure 5D) were significantly increased for diabetic wt mice and diabetic GDF15 ko mice compared to diabetic GDF15 ko controls. As of week 9, diabetic GDF15 ko mice showed a further increase in both water intake and urine production compared to diabetic wt mice.

Throughout the experiment, urinary glucose loss (Figure 5E) was elevated for diabetic wt and diabetic GDF15 ko mice compared to non-diabetic controls. As of week 14, diabetic GDF15 ko mice progressed to a higher level or urinary glucose loss compared to diabetic wt mice. Blood glucose (non-fasting) and HbA1C were similarly increased for both diabetic wt and diabetic GDF15 ko mice except for the final time point (week 18) in which blood glucose and HbA1c were both higher in the diabetic GDF15 ko mice (Figure 5G,H).

Additional parameters were measured at the end of the experiment at an age of 18 weeks (Table 3). Weights of both kidneys and the liver were increased in both diabetic wt and diabetic GDF15 ko over non-diabetic GDF15 ko controls. Heart weight was significantly increased over controls only in diabetic GDF15 ko mice. Cholesterol, LDL, HDL and triglyceride levels were similar for diabetic wt and diabetic GDF15 ko mice. In contrast, serum creatinine levels were significantly increased only in diabetic GDF15 ko mice.

Table 3: Animal characteristics of type 2 diabetic mice at week 18

Parameter	Non-diabetic GDF15 ko (n=6)	Diabetic wt (n=10)	Diabetic GDF15 ko (n=8)
L kidney (mg)	194.0±7.1	250.0±11.6#	268.1±19.7#
R kidney (mg)	190.5±7.7	272.0±13.5#	261.2±14.3#
Liver (mg)	1175±65	4106±498#	4439.8±576#
Heart (mg)	140.7±5.7	157.7±8.3	163.0±4.8#
MABP (mmHG)	106±5.8	89.9±5.6	100.3±4.9
UAE (ug/day)	23.9±11.1	280.1±72#	298.6±55#
Alb/Creat ratio (ug/mg)	58.8±17	226.3±81.5	203.4±33.6#
Cholesterol (mmol/l)	2.6±0.3	5.8±0.6#	5.5±0.8#
LDL (mmol/l)	0.2±0.0	0.3±0.1	0.3±0.1
HDL (mmol/l)	1±0.2	1.8±0.3#	1.7±0.3#
Triglycerides (mmol/l)	0.5±0.1	0.7±0.2	1.4±0.7
Serum Creatinine (µmol/l)	18.5±2.4	16.0±4.0	29.6±7.0*#

Data are given as mean ± SEM, n=number of animals; L: left, R: right, MABP: mean arterial blood pressure, UAE: urine albumine excretion; Alb/Creat ratio: urine albumin to creatinine ratio; LDL: Low-density lipoprotein; HDL: High-density lipoprotein (* $p<0.05$ diabetic wt vs diabetic GDF15 ko mice, # $p<0.05$ vs non-diabetic control GDF15 ko mice)

Interstitial and tubular damage in type 2 diabetic mice.

To assess diabetic interstitial damage in the kidney, we measured interstitial α-SMA expression by immunohistology. Interstitial α-SMA was expressed in both diabetic wt and diabetic GDF15 ko mice at 18 weeks of age (Figure 6B,C, respectively). Quantification of α-SMA staining by morphometric analysis demonstrated increased interstitial α-SMA expression in diabetic GDF15 ko and diabetic wt over non-diabetic GDF15 ko controls (Figure 7A). Diabetic GDF15 ko showed a further increase in α-SMA staining.

KIM-1 staining (Figure 6D,E,F) was confined to tubules and was most pronounced in kidneys of diabetic GDF15 ko mice (Figure 6F). Morphometric quantification demonstrated significantly increased KIM-1 expression in diabetic GDF15 ko over diabetic wt mice and non-diabetic GDF15 ko controls (Figure 7B).

PAS staining (Figure 6G-I) and subsequent morphometric analysis demonstrated increased glomerulosclerosis in both diabetic GDF15 ko and diabetic wt mice over non-diabetic GDF15 ko controls with no difference between diabetic GDF15 ko and diabetic wt mice.

The tubular damage score assessed on PAS stained sections was significantly increased only in the diabetic GDF15 ko mice (Figure 7D).

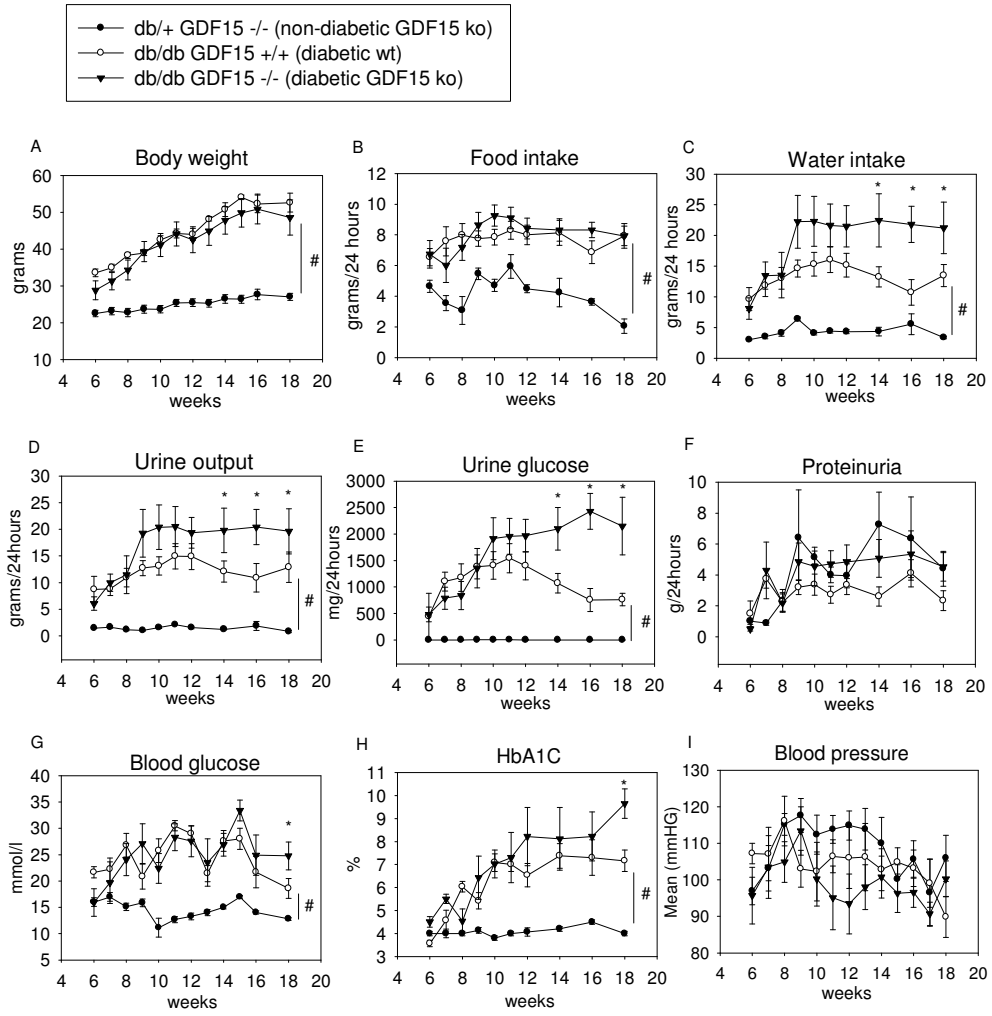

Figure 5: Effect of genomic deletion of GDF15 on parameters of type 2 diabetes (* $p<0.05$ diabetic wt vs diabetic GDF15 ko, # $p<0.05$ diabetic mice vs non-diabetic GDF15 ko mice; in panels A,B,C,D significant throughout the experiment, panel E as of week 7, panel G throughout the experiment for diabetic wt and as of week 8 for diabetic GDF15 ko, panel H as of week 8 for diabetic wt and as of week 9 for diabetic GDF15 ko). I) Blood pressure was significantly different between groups.

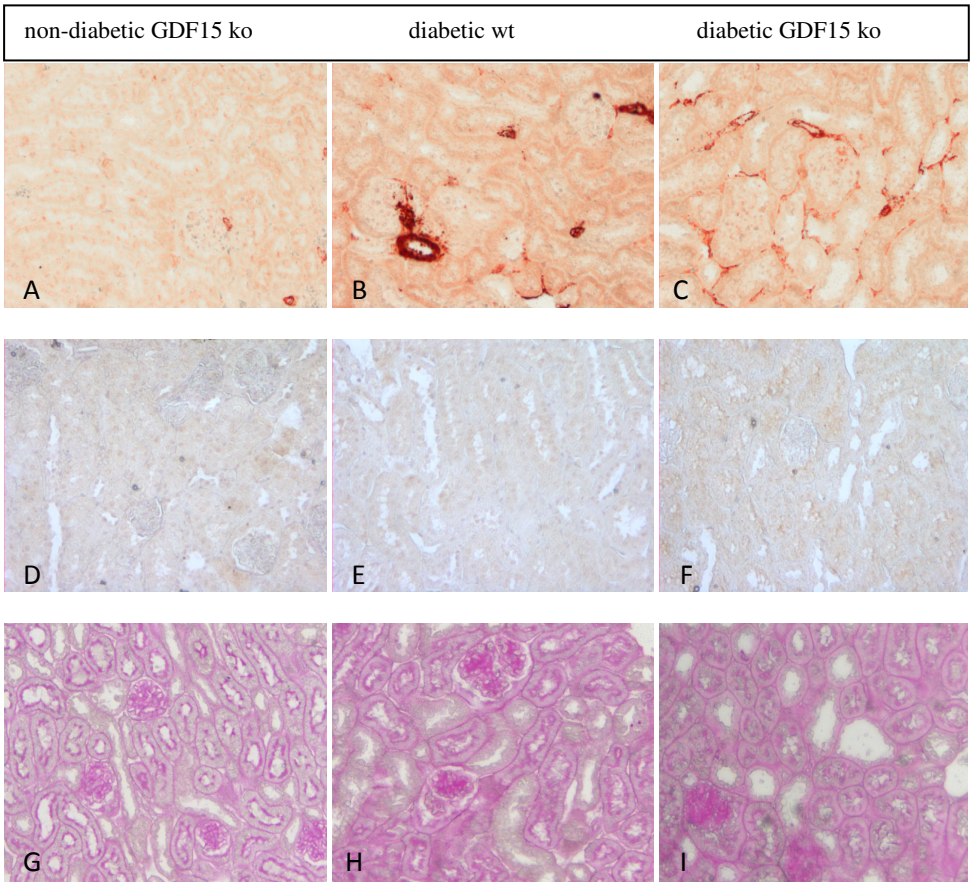

Figure 6: Representative pictures from immunohistology, showing (A-C) interstitial α-SMA expression, (D-F) KIM-1 expression in tubular epithelial cells, (G-I) PAS staining. Pictures from GDF15 ko control mice are shown in the first panel.

Taken together, these results demonstrate that diabetic GDF15 ko mice have substantially more tubular damage than diabetic wt mice at week 18 of age. In contrast, diabetic glomerular damage is not affected by genetic deletion of GDF15.

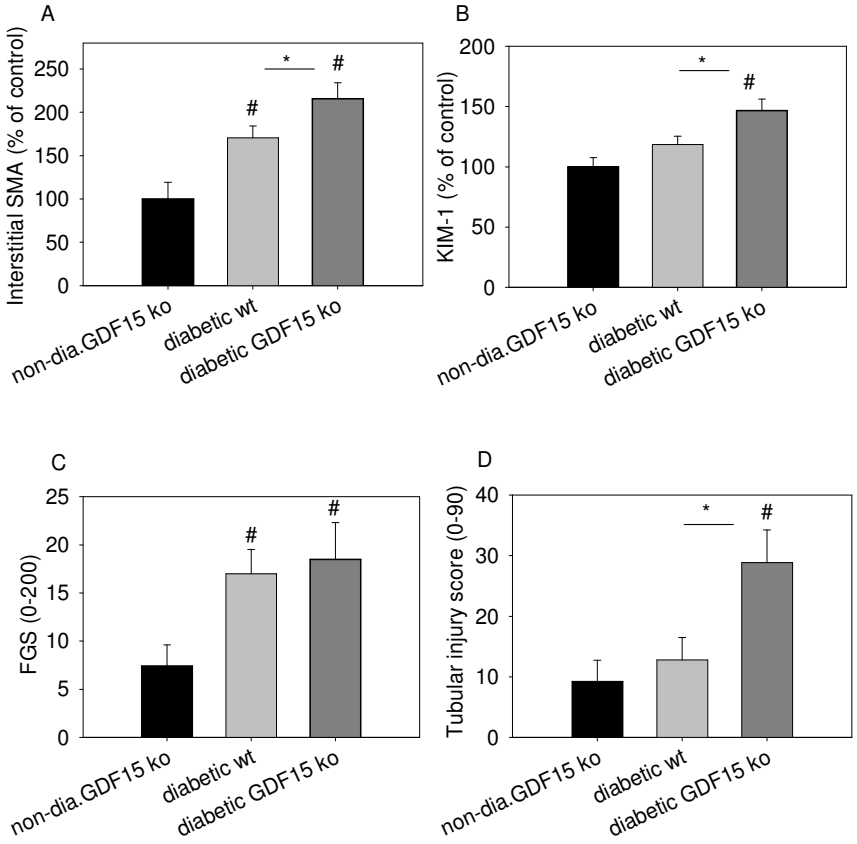

Figure 7: Interstitial pre-fibrosis (α-SMA), tubular damage (KIM-1) and kidney damage (PAS staining). (A) Morphometrical analysis of α-SMA staining in diabetic kidneys showed significantly more α-SMA expression in the diabetic GDF15 ko mice compared to non-diabetic GDF15 control and diabetic wt mice. (B) Increased KIM-1 expression was found in the diabetic GDF15 ko mice. (C,D) Renal morphology showed significant increase of FGS and tubular damage in the diabetic GDF15 ko mice compared to non-diabetic GDF15 control mice. (* $P < 0.05$ diabetic GDF15 ko versus diabetic wt mice, # $P < 0.05$ diabetic mice vs non-diabetic GDF15 ko control mice)

Renal damage markers in type 2 diabetic mice.

In addition to immunohistochemistry of KIM-1, we quantified KIM-1 mRNA expression by real-time PCR (Figure 8A). KIM-1 expression was significantly increased over controls only in diabetic GDF15 ko mice. Type 1 collagen expression was increased in diabetic wt mice with a further increase in diabetic GDF15 ko mice, although this did not reach statistical difference (Figure 8B). In diabetic wt mice, expression of podocyte markers nephrin and podocin were increased over non-diabetic GDF15 ko controls (Figure 8C,D). In diabetic GDF15 ko mice, only podocin expression was increased over non-diabetic GDF15 ko controls.

To assess renal inflammation, we measured the expression of chemokine KC, intracellular adhesion molecule ICAM-1 and macrophage inflammatory protein MIP-2 (Figure 8E, F, G). The expression of KC did not differ between groups. ICAM1 expression was significantly decreased in both diabetic wt and diabetic GDF15 ko mice. MIP-2 expression was highest in diabetic GDF15 ko mice, but did not reach significant levels over non-diabetic GDF15 ko controls.

Expression of renal glucose transporters in type 2 diabetic mice.

As diabetic GDF15 ko mice demonstrated increased urinary glucose excretion and increased urinary volume, we examined the renal expression of glucose transporters (Figure 8H, I, J, K). We observed decreased expression of Glut1, Glut2, SGLT1 and SGLT2 only in diabetic GDF15 ko mice.

Taken together, genetic deletion of GDF15 in a model of type 2 diabetes resulted in more renal damage in GDF15 ko mice over wt mice, as demonstrated by increased urinary glucose loss and increased urine production. These changes were accompanied by increased levels of renal damage markers in the GDF15 ko mice. Kidneys from type 2 diabetic GDF15 ko mice showed increased KIM-1 mRNA and KIM-1 protein expression over diabetic wt control, indicating more tubular damage in the diabetic GDF15 ko mice. Furthermore, plasma creatinine levels were increased only in the diabetic GDF15 ko mice, indicating impaired kidney function in the diabetic GDF15 ko mice.

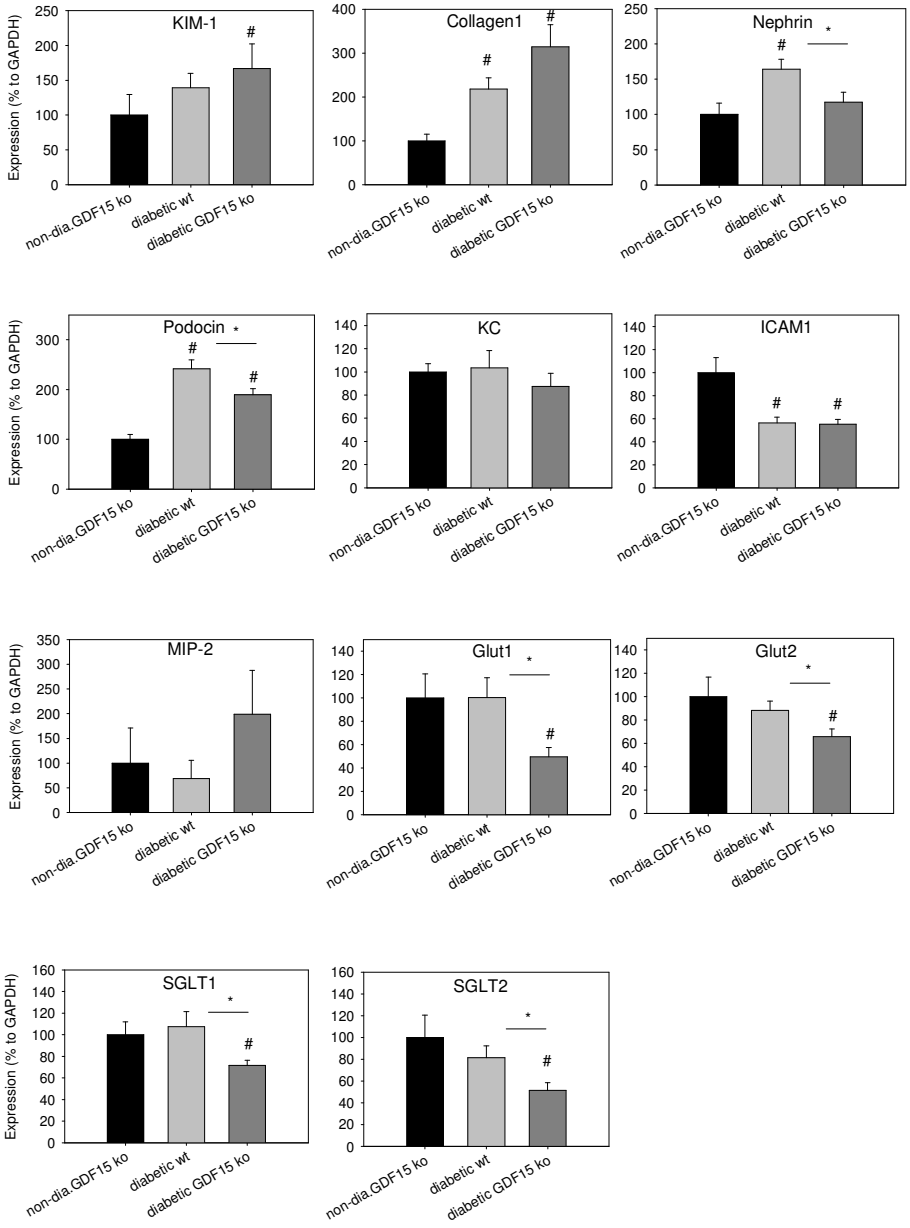

Figure 8: mRNA expression of renal markers in type 2 diabetic mice. (* p<0.05 diabetic GDF15 ko vs diabetic wt, # p<0.05 diabetic mice vs non-diabetic GDF15 ko control mice)

In-vitro effects of GDF15 on TGF-β signaling

TGF-β is central to the development of diabetes induced renal injury. As genetic deletion of GDF15 resulted in significantly increased pre-fibrosis and collagen-I expression we determined the effects of GDF15 on TGF-β signaling in cells of renal and non-renal origin. In cultured human mesangial cells, GDF15 did not stimulate the pro-fibrotic p-Smad2/3 pathway nor did it affect the anti-fibrotic p-Smad1/5/8 signaling cascade (Figure 9A,B). Cultured human mesangial cells were responsive to GDF15, as GDF15 stimulated cellular proliferation (Figure 9C). In cultured mesangial cells obtained from transgenic mice expressing GFP under the control of a Collagen 1A promoter, GDF15 did not induce Collagen 1A promoter activity nor did it reduce TGF-β induced promoter activity (Figure 9D). GDF15 did not affect p-Smad2/3 and p-Smad1/5/8 levels in mink lung epithelial cells, a cell line commonly used for its high sensitivity to TGF-β (Figure 9E,F). In addition, GDF15 did not prevent TGF-β mediated p-Smad2/3 and p-Smad1/5/8 activation (Figure 9E,F). In similar experiments performed in cultured tubular epithelial cells and NIH-3T3 fibroblast, GDF15 was also found to have no effects on Smad2/3 or Smad1/5/8 signaling (data not shown).

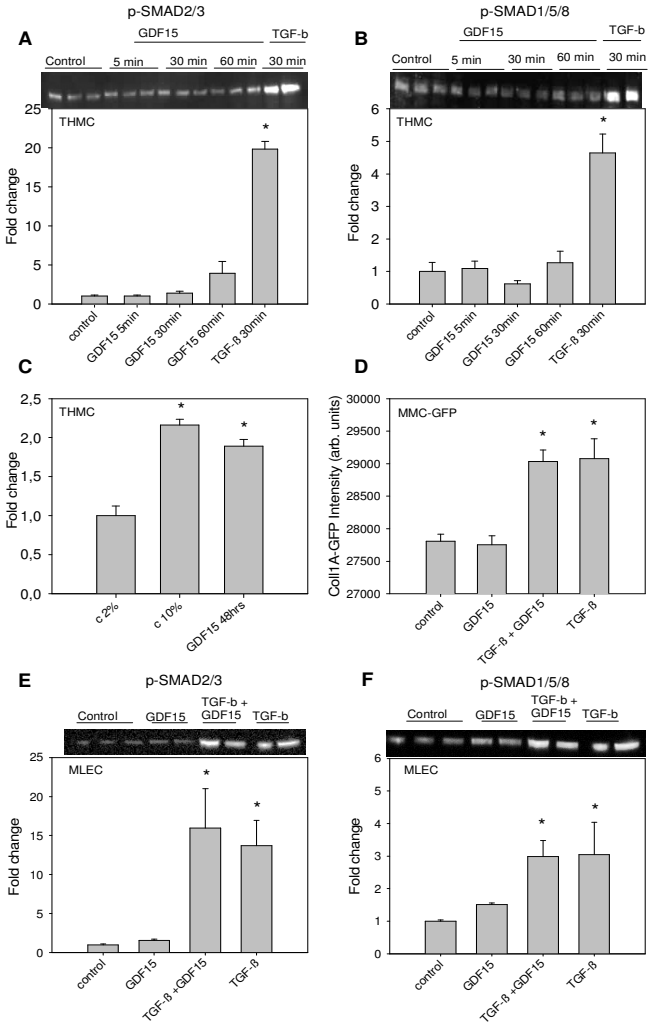

Figure 9: Effects of GDF15 on profibrotic signaling pathways in cell types responsive to TGF-ß. A,B) GDF15 did not activate p-Smad2/3 nor p-Smad1/5/8 in cultured human mesangial cells (THMC). C) Despite no effects on Smad2/3 and Smad1/5/8 signaling, GDF15 stimulated proliferation of human mesangial cells D) Mesangial cells cultured from transgenic mice expressing GFP under the control of a Collagen 1A promoter showed increased Collagen 1A promoter activity after 48 hours of stimulation with TGF-β. GDF15 did not affect Collagen 1A promoter activity E,F) GDF15 did not activate p-Smad2/3 nor p-Smad1/5/8 in mink lung epithelial cells (MLEC). GDF15 did not inhibit TGF-β stimulated Smad2/3 and Smad1/5/8 activation. (*p<0.05 relative to controls, GDF15 50ng/ml, TGF-β 10 ng/ml).

Discussion

The most striking observations in the present study investigating the role of GDF15 in diabetes is that genetic deletion of GDF15 augments renal tubular and interstitial damage in both type 1 and type 2 diabetes models. Increased interstitial and tubular damage was accompanied by excessive loss of glucose in the urine (glucosuria) and increased urine production (polyuria). Furthermore, impaired kidney function as measured by increased plasma creatinine levels was found in type 2 diabetic GDF15 knockout mice.

Despite the increased tubular damage in the GDF15 knockout mice, the glomerular sclerosis scores were similarly increased in the type 2 diabetic GDF15 knockout and wild type mice. In addition, urinary albumin excretion and albumin/ creatinine ratios were similarly increased in diabetic GDF15 knockout and diabetic wild type mice. As tubular albumin reabsorption is inhibited in diabetes[22], these changes reflect similarly increased levels of albumin leakage in the glomerulus of diabetic GDF15 knockout and diabetic wild type mice. We therefore conclude that in contrast to tubular damage, genetic deletion of GDF15 did not affect diabetic glomerular damage.

In both models, genetic deletion of GDF15 had no major effect on the severity of diabetes as blood glucose levels and HbA1C levels were similar between diabetic groups. The type 2 diabetic db/db model displayed increased blood glucose and HbA1C levels in the diabetic GDF15 knockout mice over wild type controls at the latest time-point investigated (week 18). However, as increased glucosuria in the diabetic GDF15 knockout mice was already evident as of week 14, we conclude that the augmented renal damage in GDF15 knockout mice is not caused by more severe diabetes.

Genetic deletion of GDF15 caused increased renal expression of α-SMA and collagen type 1 indicating that GDF15 may play an anti-fibrotic role in the diabetic kidney. As GDF15 is a member of the TGF-β family of signaling molecules, several studies have investigated the effects of GDF15 on Smad signaling, the signaling molecules down-stream of TGF-β. Conflicting reports on the effects of GDF15 on Smad signaling have been reported in experimental models of cardiac disease. In cultured cardiomyocytes, GDF15 activated the Smad2/3 signaling cascade, a profibrotic pathway commonly activated by TGF-β[13;15]. In contrast, a more recent paper demonstrated that GDF15 activated the Smad1/5/8 signaling cascade in cultured cardiomyocytes but not Smad2/3[13;23]. As cardiomyocytes are not the main matrix producing cells in the heart, it is likely that the observed phenomena are

associated with cardiac hypertrophy and not with organ fibrosis. In a set of *in vitro* experiments we were unable to observe a direct effect of GDF15 on either Smad2/3 or Smad1/5/8 signaling in cell types sensitive to TGF-β. These experiments included cells of renal origin (human and mouse mesangial cells and tubular epithelial cells) and non-renal cells (NIH-3T3 fibroblasts and mink lung epithelial cells). Furthermore, GDF15 was unable to inhibit TGF-β induced p-Smad2/3 signaling in these cells. Therefore, our results suggest that GDF15 modulates the renal expression of α-SMA and collagen type 1 through an indirect route and not by interfering with Smad signaling directly.

Evidence for such an indirect pathway has recently been reported in a study demonstrating that GDF15 may inhibit organ damage through controlling inflammatory cell recruitment. In this study, it was demonstrated that GDF15 inhibited leukocyte-integrin activation in experimental myocardial infarction in mice. Furthermore, GDF15 knockout mice demonstrated excessive leukocyte adhesion to the cardiac endothelium[16]. Similarly, our experiments show potentiation of the renal inflammatory response in diabetic GDF15 knockout mice over diabetic wt mice, demonstrated by increased expression of chemokine KC, adhesion molecule ICAM1 and macrophage inflammatory protein MIP-2 after STZ induced diabetes. These differences were most profound at week 1 rather than week 2 of the STZ diabetic model, suggesting that GDF15 may play an important role in preventing inflammatory cells recruitment in early diabetic inflammation of the kidney. Indeed, in the type 2 diabetic model we did not observe a similar up-regulation of these inflammatory markers at the end of the experiment.

The phenotype of the diabetic GDF15 knockout mice resembles in part those of patients with familiar renal glucosuria (FRG). These patients have impaired tubular resorption of glucose, resulting in polyuria and glucosuria. The main cause of FRG are mutations in the gene encoding for SGLT2, a critical transporter for tubular glucose resorption from the urine. The loss of excessive glucose in the urine in FRG patients has a lowering effect on blood glucose levels, resulting in a reduction of diabetic complications[24]. This condition has led to the development of drugs that specifically inhibit SGLT2.

In our experiment in type 2 diabetic mice we found a selective down-regulation in the renal expression of SGLT2 and other glucose transporters in the diabetic GDF15 knockout mice over diabetic wild type control and non-diabetic GDF15 knockout mice. As SGLT2 is the main glucose transporter to resorb glucose from the urine into the tubular cell[25], our data in

type 2 diabetic mice suggest that the observed glucosuria is caused by tubular SGLT2 down-regulation. In the type 1 diabetic model, the association between the expression of glucose transporters and the presence of glucosuria was less clear and may have been obscured by the large variation in the expression of glucose transporters within some groups.

In contrast to the FRG patients and subjects on SGLT2 inhibitors, genetic deletion of GDF15 and the associated glucosuria did not result in lower blood glucose levels and protection from diabetic complications to the kidney. Despite the increased glucosuria in GDF15 knockout mice, blood glucose levels were similarly elevated in diabetic GDF15 knockout and wild type mice in both models of diabetes. In the type 1 model, food intake was significantly increased in the diabetic GDF15 knockout over diabetic wild type and non-diabetic controls, allowing the maintenance of high blood glucose levels. In the type 2 diabetic model, food intake in the GDF15 knockout mice was not significantly higher over diabetic wild type controls. However, food consumption in the obese db/db mice is dramatically increased over non-diabetic db/+ controls throughout the experiment, providing sufficient spare capacity to maintain high blood sugar levels. Therefore, the maintenance of high blood glucose levels in the diabetic GDF15 knockout mice most likely prevents the protective effects of increased glucosuria in the GDF15 knockout mice.

Although the streptozotocin (STZ)-induced diabetic mouse model is commonly used[26], we cannot exclude that some of the effects observed in the STZ model are caused or augmented by direct toxicity of STZ to the kidney or to other organs. However similar renal changes were found in the type 2 diabetic db/db mice model. Therefore, it is unlikely that changes in direct effects of STZ on ko animals played a prominent role in the excess renal damage found in this study.

In both rats and mice, increased GDF15 expression was localized in the glomerulus. However, the tubuli and renal interstitium appear to be important targets for GDF15 as genetic deletion of GDF15 had profound effects on interstitial pre-fibrosis and tubular function and integrity. It is conceivable that the diabetic damage is detected in the glomerulus resulting in increased glomerular GDF15 production. Subsequently, glomerular GDF15 may be transported by the efferent arteriole to exert its protective effects in the renal interstitium and on the tubuli. Development of selective glomerular GDF15 knockout and knockin models may proof helpful to determine the functional effects of glomerular

GDF15 production in diabetes. Furthermore, the receptor for GDF15 has still not been identified, hampering the identification of (renal) target tissues for GDF15.

Finally, GDF15 has recently emerged as a promising clinical biomarker for cardiovascular disease and type 2 diabetes[4;27]. In these studies, higher GDF15 plasma levels were associated with increased cardiovascular morbidity and increased kidney damage. This appears to be in contrast to the protective role for GDF15 in myocardial infarction[28] and diabetic kidney disease (this study).
In the present study we showed that the diabetic glomerulus is a source of GDF15 with its targets in the tubular and interstitial compartment. Therefore plasma GDF15 levels may reflect the extent of diabetic damage in the glomerulus. Nevertheless, GDF15 provides protection to the interstitium and tubuli. Therefore the development of GDF15 agonists may provide new possibilities for the treatment of diabetic complications to the kidney in both type 1 and type 2 diabetes.

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Chapter 5

Growth Differentiation Factor 15 impairs aortic contractile and relaxing function through an endothelial action

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Leo E. Deelman

Abstract

Background

Growth Differentiation Factor 15 (GDF15) is emerging as an independent predictor of cardiovascular disease (CVD) although the functional relationship between GDF15 and CVD is not clear. Increased GDF15 levels have been associated with endothelial dysfunction in a selected patient population. We therefore investigated whether GDF15 modulates endothelial function in aortas of wild type (wt) and GDF15 knockout (ko) mice.

Methods

Vascular contractions to phenylephrine (PE) and relaxation to acetylcholine (ACh) were assessed in aortic rings obtained from healthy wt and GDF15 ko mice. In these animals, also the effects of GDF15 pretreatment on vascular function were determined. The involvement of reactive oxygen species (ROS) and caveolae was determined using tempol/ catalase and MCD, respectively.

Results

PE induced contractions and ACh mediated relaxations were similar in wt and GDF15 ko mice. Pretreatment with GDF15 inhibited contraction and relaxation in both wt and ko groups. Inhibition of contraction by GDF15 was absent in denuded vessels and after blocking NO-synthase. Relaxation in wt was mediated mainly through NO and EDHF, while GDF15 ko mice mainly employed PGs and EDHF. Pretreatment with GDF15 impaired ACh mediated relaxation in wt mice by decreasing NO, while in GDF15 ko mice this was mediated by decreased action of PGs. Disruption of caveolae resulted in a similar inhibition of vascular responses as GDF15 and administration of GDF15 did not result in a further inhibition of vascular responses. ROS inhibition did not affect vascular function.

Conclusion

GDF15 impairs aortic contractile and relaxing function through an endothelial dependent mechanism.

Introduction

Endothelial dysfunction is widely recognized as a key event leading to cardiovascular disease and is defined as an imbalance between vasodilating and vasoconstricting substances produced by the endothelium[1;2]. In addition, endothelial dysfunction is associated with increased adhesion molecule expression and reduced anticoagulant properties[3].

Growth differentiation factor 15 (GDF15), a distant member of the Transforming Growth Factor-β family and an early response gene, is emerging as an independent predictor of cardiovascular disease in the elderly and a predictor of prognosis in patients with established cardiovascular disease[4-8]. In an elderly population an association between GDF15 levels and endothelial dysfunction was found[8]. Furthermore, a recent study demonstrated an effect of GDF15 on endothelial adhesion molecule expression, as GDF15 inhibited leukocyte recruitment and inflammation by inhibiting endothelial integrin activation in an experimental model of myocardial infarction[9].

These data suggest a possible role for GDF15 in the development of endothelial dysfunction in cardiovascular disease. Therefore, we investigated the effects of genetic deletion of GDF15 and the effects of acute treatment with recombinant GDF15 on vascular responsiveness of mouse aorta. For this, vascular contractility and endothelium mediated relaxation was assessed in aortic rings of wt and GDF15 ko mice.

Materials and methods

Animal model

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen (Groningen, the Netherlands). The basic study was performed in 7 male mice (12-14 weeks old, 25.1- 29.4 grams; C57BL/6, Harlan, The Netherlands) and 7 male GDF15 ko mice (12-14 weeks old, 22.6- 25.3 grams, from internal breeding, 5 breeding pairs of GDF15 ko mice on a C57BL/6 background were obtained from Johns Hopkins University Baltimore, Maryland, USA) housed under standard conditions with free access to food and drinking water throughout the study. Supplement vascular data were obtained from additional 19 male mice (10-14 weeks old, 24- 31 grams; C57BL/6, Harlan, The Netherlands). WT and ko mice were anesthetized with isoflurane (2%) and the thoracic aorta was removed and temporarily stored in cold physiological saline.

Vasomotor responses

Freshly isolated thoracic aortic rings (1.5 - 2 mm in length) were mounted on 200μm stainless wires in individual myograph baths (Danish Myo Technology A/S, Aarhus, Denmark) as described previously [10]. Briefly, baths containing 6ml of Kreb's solution were warmed to 37°C and pre-equilibrated continuously with 95%O_2/5%CO_2 to maintain pH at 7.4. Length of aortic strips was assessed by microscopy. Aortic rings were equilibrated for 40 min until they were at a steady baseline. Then, rings were subjected twice to stimulation with potassium chloride KCl (60 mM) in order to obtain reproducible contractile responses. After stabilization, each ring was treated for 20 minutes with different compounds: control, TGF-β treatment (3 ng/ml) or GDF15 treatment (50 ng/ml). Contraction responses were measured as cumulative concentration response curves to KCl (10 mM-90 mM) and PE (10 nM-100 μM). Indomethacin (10 μM) or L-NMMA (1 μM) was used to identify the contribution of specific endothelium derived relaxing factors. Endothelium-dependent relaxation was measured by obtaining concentration-response curves to ACh (10 nM-300 μM) in rings precontracted with PE (1 μM). The influence of vasoactive prostanoids on the response to ACh was examined by incubating rings with the non-specific cyclooxygenase inhibitor indomethacine (10 μM) administered 20 min before application of PE. The influence of nitric oxygen (NO) was examined by incubating rings with indomethacine (10 μM) and a NO synthesis inhibitor L-N^G-monomethyl arginine (L-NMMA) (1 μM) administered 20 min before application of PE. At the end of each experimental protocol, endothelium-independent relaxation was measured by applying the

nitric oxide-donor sodium nitroprusside (0.1 mM). In some experiments, the endothelial cell layer was removed by rubbing the luminal side of the vessel with a moistened wire. Non-denuded or denuded aortas were treated for 20 minutes with control or GDF15 pretreatment.

Involvement of ROS or caveolae in endothelium-dependent relaxation of the aorta

To investigate the involvement of ROS and caveolae on endothelium-dependent relaxation of the aorta, aortic rings were preincubated for 40 minutes with the membrane-permeable radical scavengers tempol (100 μmol/l) and catalase (500 U/ml) or with the caveolae disrupting agent methyl-β-cyclodextrin (MCD, 1mM). ACh dose response curves were obtained as described above.

Drugs

Krebs solution was prepared freshly and of the following composition (mM): 120.4 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.2 NaH_2PO_4, 11.5 glucose; all components obtained from Merck (Darmstadt, Germany). Stock solution (10 mM) for indomethacine, was prepared in 96% ethanol. Tempol, freshly prepared catalase, and MCD were dissolved in saline solution. All other drugs were dissolved in deionized water. NG-Monomethyl-L-arginine acetate salt was purchased from MP Biomedicals (Illkirch, France). Recombinant human TGF-β1 and GDF15/MIC-1 were obtained from Peprotech and dissolved in PBS. All other compounds were purchased from Sigma. The concentrations presented in the concentration-curve responses are expressed as final molar concentrations in the organ baths.

Calculations and statistical analysis

Concentration-response curves to ACh and SNP were expressed as percentage relaxation of preconstriction obtained with PE. Concentration-response curves to ACh were characterized by calculation of the Area Under the Curve (AUC). The AUC was used to present total ACh relaxation, first in the absence of indomethacin for total ACh responses, later in the presence of indomethacin for prostaglandin contribution. Data are present as mean ± S.E.M. and n refers to the number of animals in each group. Concentration-response curves of individual rings were plotted with SigmaPlot version 10.0 (Systat Software Inc, San Jose, CA). Statistical analysis was done with SPSS 16.0.2 for Windows (SPSS Inc., Chicago, Illinois). Differences between full dose-response curves were tested

with repetitive ANOVA, differences between points with one-way ANOVA. A probability (p) value of <0.05 was considered statistically significant.

Results

Effects of genetic deletion of GDF15 on vascular function.

To investigate the potential effects of GDF15 on vascular function, we first compared the vascular function of healthy wt and GDF15 ko mice. Animal characteristics are listed in Table 1. GDF15 ko mice had lower body weights, increased left and right kidney weights and decreased liver weight compared to wt controls.

Table 1: Animal characteristics

	Wt (n=7)	GDF15 ko (n=7)
Age (weeks)	14±0,4	14±0,8
Body weight (g)	26,6±0,4	24,6±0,2#
L kidney (mg)	178±5	196±8#
R kidney (mg)	189±6	207±8#
Liver (mg)	1415±50	1209±52#
Heart (mg)	150±7	145±6
liver/BW (mg/g)	53.3±1.9	49±2.0
heart/BW (mg/g)	5.7±0.2	5.9±0.2

All data are presented as mean ± SEM

#p<0.06 GDF15 ko vs. wt control mice

Pretreatment with GDF15 impairs contractile reactivity.

Contractile responses of aortic rings were obtained by constructing cumulative concentration response curves to PE (PE, figure 1A,B). PE induced contraction of wt and GDF15 ko mice did not differ with respect to EC_{50} and maximum response (E_{max}). Pretreatment with GDF15 (50 ng/ml, for 30 minutes) induced a significant reduction in the response to PE both in wt and GDF15 ko animals. To investigate whether the vascular effects of GDF15 were distinct from those of TGF-β, additional rings were pretreated with a high dose of TGF-β (3ng/ml). TGF-β did not affect the PE response (Figure 1A,B). As GDF15 pretreatment also inhibited the response to 100μM KCl following the PE concentration response curve in both wt and ko mice (Figure 1A,B), we investigated the

effect of GDF15 on a concentration response curve to KCl in wt mice. GDF15 impaired KCl responses at 30 mM (con: 1.7±0.2 mN, GDF15: 0.9±0.1 mN), 60mM (con: 3.8±0.3mN, GDF15: 2.9±0.3 mN and 90 mM (con: 4.6±0.4 mN, GDF15: 3.9±0.3 mN).

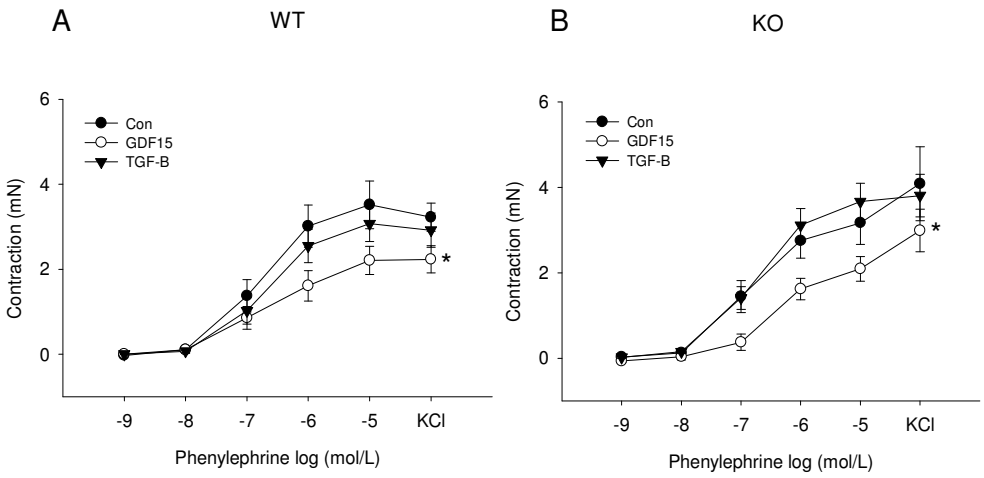

Figure 1: GDF15 impairs aortic contraction to PE in wt and GDF15 ko mouse. A) PE concentration response curve in wt mice aortic rings. B) PE concentration response curve in GDF15 ko mice aortic rings. n=7 mice per condition. *P<0.05 control (con) vs GDF15 treatment.

GDF15 inhibition of vascular contraction is endothelium-dependent and involves the NO pathway.

To investigate the mechanism through which GDF15 impairs vascular contraction, PE induced vascular contraction was assessed in aortic rings after removal of the endothelium. In aortas with intact endothelium, pretreatment with GDF15 again resulted in impaired PE mediated contraction (Figure 2A). In contrast, denudation of aortic rings abrogated the inhibition of PE contraction by GDF15, indicating the requirement of endothelium for the GDF15 mediated inhibition of PE contractility.

To further investigate which endothelium derived factors are required for the GDF15 mediated inhibition of vascular contractility, (non-denuded) aortas were incubated with compounds that inhibit nitrous oxide release (L-NMMA) and prostaglandin synthesis (indomethacine). Pretreatment with L-NMMA completely blocked the inhibitory effects of

GDF15 on vascular contractility (Figure 2B), whereas pretreatment with indomethacine did not affect the GDF15 mediated impaired vascular contractility (data not shown).

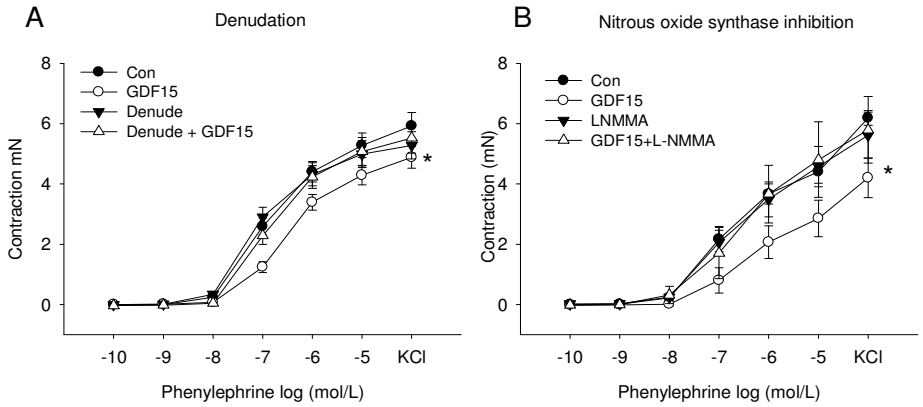

Figure 2: GDF15 inhibition of vascular contraction is endothelium-dependent and involves the NO pathway. A) Concentration-response curves to PE in intact or endothelium-denuded mice aortic rings obtained from wt mice. n=17 rings for intact aortas, n=14 rings for denudated aortas. B) Abrogation of GDF15 effect by inhibition of NO-synthase inhibitor by L-NMMA, n=5-6 for all groups. Data are expressed as means ± SEM. *P<0.05 vs control (con).

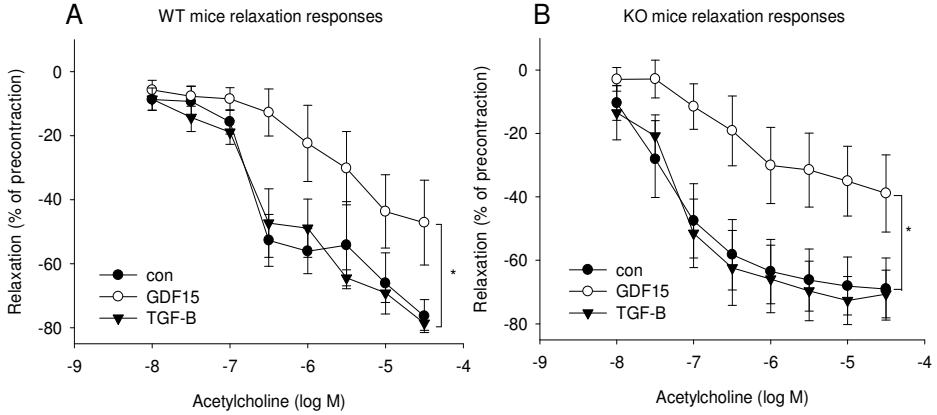

Figure 3: GDF15 inhibits endothelium-dependent induced relaxation of PE precontracted aortic rings in wt (A) and GDF15 ko mice (B). Data are expressed as means ± SEM. n=5-7 per condition. *P<0.05 control (con) versus GDF15 pretreatment.

GDF15 inhibits endothelium-dependent relaxation in wt and GDF15 ko mice.

To investigate the effects of GDF15 on endothelium dependent relaxation of the aorta, we compared ACh induced vascular relaxation in wt and GDF15 ko mice. For this, rings were precontracted with PE and cumulative dose-response curves to ACh were constructed (Figure 3). Dose-response curves to ACh were similar for wt and GDF15 ko mice. Pretreatment of the aortas with GDF15 caused a strong impairment of the ACh induced relaxation in aortas of both wt and GDF15 ko mice. To investigate whether the effects of GDF15 on relaxation were distinct from those of TGF-β, we included rings that were pretreated with TGF-β. TGF-β did not affect the ACh mediated relaxation.

The contribution of prostaglandins (PGs), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) to endothelium-dependent relaxation

To investigate the relative contribution of the individual endothelium derived relaxing factors, dose-response curves to ACh were constructed in the presence of indomethacine and the combination of indomethacine and L-NMMA (Figure 4). The relative contribution was defined as the relative Areas Under the Curve (AUC). In wt mice, the total Area Under the Curve was 141±13.6 (%*log(M)). AUCs for PGs, NO and EDHF amounted 15±13, 50±21 and 90±14, respectively, indicating that vascular relaxation in wt mice was mediated mainly through NO and EDHF (Figure 4A).

In GDF15 ko mice, the total Area Under the Curve was 116±30 (%*log(M)). AUCs for PGs, NO and EDHF were 59±41, 14.5±33 and 31±45, respectively, indicating that vascular relaxation in GDF15 ko mice was mediated mainly through PGs and EDHF (Figure 4C). Therefore, the total ACh mediated relaxation was similar for wt and GDF 15 ko mice, however the relative contribution of the relaxing factors differs.

Pretreatment of the aorta rings with GDF15 resulted in impaired relaxation as indicated by a decreased total Area Under the Curve in both wt (104±18) and GDF15 ko (62±27) (Figure 4B,D). In GDF15 pretreated wt mice, the impaired relaxation was mainly mediated by inhibition of the NO component (Figure 4B). In GDF15 ko mice the impaired relaxation after pretreatment with GDF15 appeared to be caused by inhibition of PGs (Figure 4D). However due to the larger variation in the data obtained the decrease in endothelial components did not reach significant levels in GDF15 ko mice.

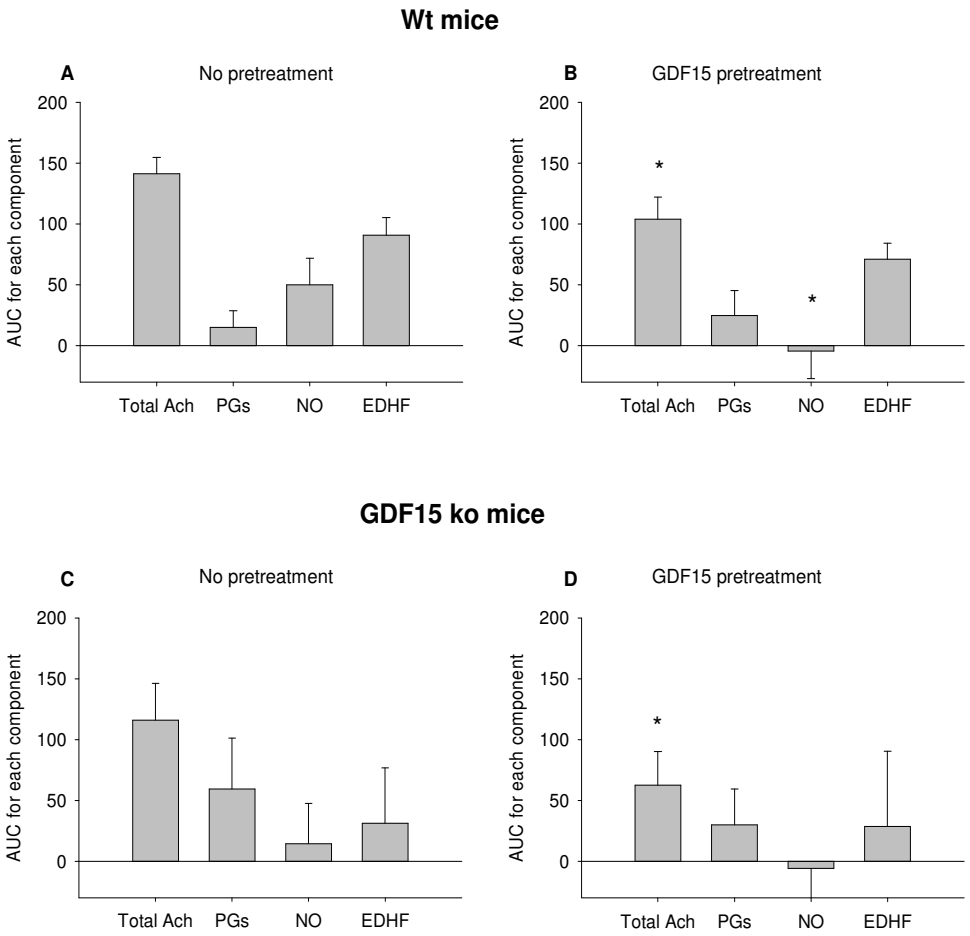

Figure 4: Differences in contribution of endothelial components in endothelium-dependent relaxation of aortas of wt and GDF15 ko mice without and with pretreatment with GDF15.

ACh-mediated relaxation of PE precontracted aortic rings from wt mice (A,B) and GDF15 ko mice (C,D). After indomethacine and L-NMMA treatment the contribution of prostaglandins (PGs), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) on endothelial function were calculated. Data are expressed as means ± SEM. n=5-7 per condition (*$p<0.05$ vs corresponding group without GDF15 pretreatment).

Vascular responses to sodium nitroprusside.

To investigate potential differences in the sensitivity of smooth muscle cells to NO, we measured the effects of the NO donor sodium nitroprusside (SNP) on vascular relaxation. The relaxant response to SNP was similar in all groups (Table 2), indicating that the impaired relaxation after GDF15 pretreatment was not caused by differences in NO sensitivity of the vascular smooth muscle cells.

Table 2: Maximal relaxation to SNP (% of PE contraction)

	WT		GDF15 ko	
Treatment	**n**	**SNP**	**n**	**SNP**
control	6	98,10±0,86	7	98,28±1,78
GDF15	7	89,76±4,5	7	100,92±7,43

Data are expressed as a percentage of PE-mediated precontraction and were obtained using 0.1mM of SNP. No differences were found between groups (one-way ANOVA).

Effects of ROS inhibition and caveolae disruption on aortic responses

To investigate the mechanism through which GDF15 impairs vascular contraction and relaxation, we evaluated the involvement of reactive oxygen species and caveolae. For inhibition of reactive oxygen species, vascular segments were incubated with tempol and catalase (TC). For disruption of caveolae, aortas were pretreated with MCD.

As in previous experiments, GDF15 inhibited PE mediated contraction, resulting in a significantly reduced AUC (Figure 5A, black bars). TC pretreatment did not affect PE mediated contraction, nor did it affect the GDF15 with TC mediated inhibition of PE induced contraction (Figure 5A, white bars). Pretreatment with MCD resulted in significantly lower PE mediated contraction. Importantly, pretreatment with MCD precluded the GDF15 mediated reduction in PE contraction (Figure 5A, grey bars).

Similar effects of ROS inhibition and caveolae disruption were observed on ACh mediated relaxation. GDF15 inhibited ACh induced relaxation, resulting in a significantly decreased AUC (Figure 5B, black bars). Pretreatment with TC did not affect ACh mediated relaxation and GDF15 inhibited ACh mediated relaxation in the presence of TC (Figure 5B, white bars). MCD lowered ACh mediated relaxation, although this did not reach statistical significance (Figure 5B, grey bar). GDF15 did not further reduce ACh mediated relaxation in MCD pretreated rings.

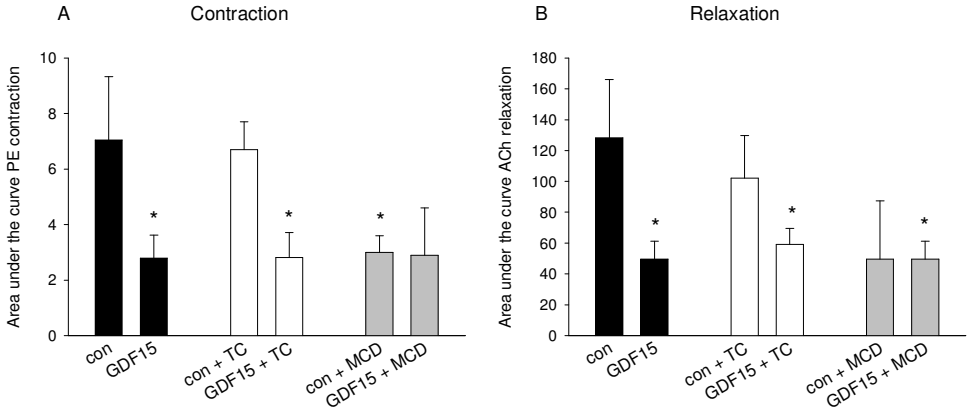

Figure 5: Disruption of caveolae, but not inhibition of ROS, results in a similar inhibition of vascular contraction and relaxation as GDF15. AUCs were calculated for PE dose response curves (A) or ACh relaxations (B) of aortic rings from wt mice. Relaxation was expressed as percentage of preconstriction. Data are expressed as means ± SEM. n=5 mice per condition. *p<0.05 vs control (con)

Discussion

The main findings of the present study is that GDF15 modulates endothelial function resulting in impaired vascular contraction and relaxation in murine aortic rings. The effects of GDF15 on the endothelium include increased basal NO release and impaired release of endothelial derived relaxation factors in response to ACh. Disruption of caveolae by MCD had similar inhibitory effects on vascular function as GDF15. Furthermore, MCD treatment abrogated the inhibitory effect of GDF15 both on contraction and relaxation, indicating that GDF15 may inhibit endothelial function through a caveolae dependent mechanism.

Our data demonstrate that GDF15 mediated inhibition of PE induced aortic contraction is most likely caused by the induction of endothelial release of NO. First, removal of the endothelium resulted in abrogation of the inhibitory effects of GDF15 on aortic contraction. Furthermore, in intact aortic rings, the inhibitory effects of GDF15 was blocked by pretreatment with the NO-synthase blocker L-NMMA. Finally, contraction in responses to KCl was similarly inhibited as for PE, indicating that the inhibition of vascular contraction by GDF15 was not specific for the adrenergic receptor system. We therefore conclude that GDF15 inhibits aortic contraction and that this mechanism most likely involves enhancement of basal NO release by GDF15.

In addition to its effects on contraction, an even more profound action of GDF15 was found with respect to relaxation. Pretreatment with GDF15 inhibited acetylcholine (ACh) induced relaxation of aortic rings that were precontracted with PE in both wt and GDF15 ko mice. As levels of relaxation were similar in control and GDF15 treated rings upon addition of the NO-donor sodium nitroprusside, GDF15 impairment of relaxation was not caused by possible effects on PE mediated preconstriction. Furthermore, sodium nitroprusside experiments indicate that the impaired relaxation to ACh was not caused by differences in the sensitivity of smooth muscle cells to NO.

Identification of the components responsible for the GDF15 mediated impaired relaxation to ACh, demonstrated that GDF15 inhibited the NO component in aortic rings obtained from wt mice. In GDF15 ko mice, the inhibited relaxation appeared to be caused by a decreased contribution of the prostaglandin and NO component, but this did not reach statistical significance.

Taken together, our data demonstrates that GDF15 modulates endothelial function by increasing basal NO release, while inhibiting the release of relaxing factors after stimulation with ACh. These actions resulted in impaired contraction and impaired relaxation in aortic rings obtained from wt and GDF15 ko mice.
The mechanism of action through which GDF15 exerts these effects remain elusive. As an obvious option, GDF15 may act on receptors of the TGF-β superfamily[11]. However, in contrast to GDF15, TGF-β did not affect PE mediated contraction or ACh mediated relaxation, precluding an effect of GDF15 through TGF-β type 1 and 2 receptors.

Alternatively, given its opposite effects on unstimulated and stimulated endothelium, GDF15 may affect a general mechanism influencing endothelial cell signal transduction, including the generation of reactive oxygen species (ROS) signaling and caveolae dependent signaling. While ROS are known for the ability to react with and neutralize NO[12], it is unlikely that modulation of this pathway is employed by GDF15, as tempol and catalase did not affect inhibition of vascular responses by GDF15. In contrast, GDF15 seems to affect caveolar signaling as disruption of caveolae resulted in a similar inhibition of vascular responses as GDF15 and administration of GDF15 did not result in a further inhibition of vascular responses.

Our current study demonstrates that GDF15 may impair PE mediated contraction through a mechanism that involves increased basal NO-release by the endothelium. Several studies have shown increased basal NO production in cells deficient for caveolin-1, a protein essential for caveolae formation[13]. Furthermore, a recent study demonstrated that the scaffolding domain of caveolin-1 acts as an endogenous negative regulator of e-NOS function where disruption of the scaffolding domain resulted in increased basal NO release[14]. Therefore, our data suggests that pretreatment with GDF15 may affect the interaction of caveolin-1 with eNOS, resulting in enhanced basal NO-production under non-stimulated conditions.
In contrast to non-stimulated conditions, a previous study by our group demonstrated that caveolar disruption impaired ACh stimulated NO and EDHF release in aorta and other arteries in rat [13]. Furthermore, the enzymes involved in prostaglandin release were found to be associated with caveolin-1, implicating a role for caveolae in prostaglandin release[15]. Therefore our data suggests that GDF15 may inhibit vascular relaxation by NO and other relaxing factors through a mechanism that involves modification of caveolar signaling of the endothelium.

The mechanism through which GDF15 modulates eNOS activity and caveolar function is unclear. However, for the related cytokine TGF-beta it was demonstrated that the TGF-beta receptor type 1 interacted with eNOS in the caveolae of human endothelial cells and had a regulatory function on basal eNOS enzymatic activity[16]. As the receptor(s) for GDF15 are still unknown, this mechanism can not yet be confirmed for GDF15.

Our current study demonstrates that GDF15 has pronounced effects on endothelial function, resulting in impaired contraction and relaxation. Nevertheless, genetic deletion of GDF15 is not associated with major hemodynamic effects in GDF15 ko mice Concentration-response curves to PE and KCl were similar in wt and GDF15 ko mice, indicating that genetic deletion of GDF15 does not affect aortic responses to PE and KCl. Furthermore, the inhibitory effects of GDF15 on vascular contraction was not affected by genetic deletion of GDF15, indicating that the receptor signaling cascades for GDF15 remain active in the absence of GDF15. In addition, concentration response curves of ACh mediated relaxation were similar in wt and GDF15 ko animals, indicating that genetic deletion of GDF15 did not have a marked effect on total ACh mediated relaxation. However, inhibition of the individual components of endothelium derived relaxing factors demonstrated that wt mice employed primarily the NO and EDHF pathways, while GDF15 ko mice primarily used the prostaglandin and EDHF pathways in ACh mediated relaxation of aortic rings.

The absence of a clear vascular phenotype of GDF15 ko mice despite the strong vascular effects of GDF15 may be explained by the observation that GDF15 expression is generally low in healthy animals and GDF15 expression is only induced after tissue damage[17-20]. We therefore performed a provisional experiment in type 2 diabetic db/db mice, an established model with endothelial dysfunction[21-23]. Genetic deletion of GDF15 in the db/db model prevented the impairment of ACh mediated aortic relaxation (unpublished data), indicating a role for GDF15 in endothelial dysfunction in diabetes. However, genetic deletion of GDF15 also had multiple effects outside the vasculature, including increased diabetic kidney damage. Therefore, we cannot exclude that indirect effects of genetic deletion of GDF15 resulted in the prevention of impaired ACh mediated relaxation in diabetes. Development of tissue-specific GDF15 ko mice and identification of the still unknown receptors for GDF15 will contribute to a better understanding of the role of GDF15 in the development of endothelial dysfunction in models of cardiovascular disease.

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Chapter 6

Growth Differentiation Factor 15 predicts worsening of albuminuria in patients with type 2 diabetes

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Abstract

Background

Development of micro- or macroalbuminuria is associated with increased risk of cardio-renal complications, particularly in diabetes. For prevention of transition to micro- or macroalbuminuria more accurate prediction-markers on top of classical risk-markers are needed. We studied a new promising marker, Growth Differentiation Factor 15 (GDF15), to predict transition to increasing stage of albuminuria in type-2 diabetes (T2DM). In addition, we looked at the GDF15 potential in non-diabetic subjects with hypertension (HT).

Methods

Cases and controls were selected from the PREVEND-cohort, a large (n=8,592), prospective general population study on the natural course of albuminuria, with >10 years of follow-up and repeated albuminuria-measurements. We found 24 diabetic and 50 hypertensive cases transitioning from normo to macro, and 9 diabetic and 25 hypertensive cases from micro to macro (average follow-up 2.8 yrs). Controls with stable albuminuria were pair-matched for age, gender, albuminuria-status and diabetes-duration. GDF15 was measured in samples prior to albuminuria-transition.

Results

Prior to transition, GDF15 was significantly higher in cases with T2DM than in controls (1288[885-1546] vs. 948[660-1016] pg/mL,P<0.001). The odds-ratio for transition in albuminuria increased significantly per SD of GDF15 (2.9 [95%CI 1.1-7.5],P=0.03). GDF15 also improved prediction of albuminuria-transition, with significant increases in C-statistic (from 0.87 to 0.92, P=0.03) and integrated-discrimination-improvement (0.148, P=0.001). In HT, GDF15 was also independently associated with transition in albuminuria-stage (2.0[1.1-3.5], P=0.02) and improved prediction significantly.

Conclusions

We identified GDF15 as a clinically valuable marker for predicting transition in albuminuria-stage in T2DM beyond conventional risk markers. These findings were confirmed in non-diabetic hypertensive subjects.

Intorduction

The prevalence of chronic kidney disease (CKD) is increasing and has become a major public health challenge[1]. This increase in CKD is largely due to the rapidly expanding epidemic of type 2 diabetes mellitus (T2DM) leading to diabetic nephropathy and ultimately end-stage renal disease (ESRD)[2;3]. Transition to increasing stages of albuminuria (i.e. normo- to microalbuminuria and micro- to macroalbuminuria) is considered a hallmark of progression of renal disease in diabetes[4].

However, once transitioned from normo to microalbuminuria or to macroalbuminuria, regression of disease is very difficult to achieve. Indeed, recent trials in normo-, micro-, and macroalbuminuric diabetic subjects showed that early intervention (in normoalbuminuric stage) is more effective with the same intervention. Early markers that detect those that have an increased risk for developing micro- or macro- albuminuria could thus help us reduce the number of patients at renal risk through selective preventive treatments of such patients[5;6].

Many risk factors have been linked to transition from normo- to micro and micro- to macroalbuminuria, such as hyperglycemia, hypercholesterolemia and hypertension[7;8]. However, accurate risk stratification of subjects at risk remains challenging. Novel biomarkers may help to improve the identification of subjects at risk, as well as improve insight in the underlying pathophysiology of the development of micro- or macroalbuminuria. Whereas several promising novel biomarkers were described in literature, this had not led to improved risk stratification in T2DM[9].

The lack of well-designed prospective studies that first stored samples of individuals for novel risk marker analyses and then followed the course of albuminuria over time may explain the paucity of knowledge on the prognostic value of novel biomarkers to improve risk stratification. We performed a nested case-control study in the large general population cohort ‘Prevention of REnal and Vascular End-stage Disease’ (PREVEND) to investigate novel biomarkers that may precede and predict the transition in albuminuria

Growth Differentiation Factor 15 (GDF15), a member of the TGF-β family, is a promising novel biomarker, which has been implicated as predictor for cardiovascular and all-cause mortality[10]. Interestingly it was also associated with renal outcome and a faster decline of eGFR as well as mortality in type 1 diabetic patients with macroalbuminuria[11]. It is unclear

whether these findings regarding renal outcome are also applicable to patients with type 2 diabetes. In the current study, we investigated whether circulating GDF15 levels precede and predict the development of micro- or macroalbuminuria in type 2 diabetic patients. To test wheter this is specific to diabetes, we performed a replication study to assess the predictive value of GDF15 in non-diabetic hypertensive patients.

Patients and methods

The present study was performed as a nested case-control study in subjects participating in the 'Prevention of REnal and Vascular End-stage Disease' (PREVEND) study. This prospective community-based cohort study on the natural course of urinary albumin excretion with serial follow-up measurements was initiated in 1997. Details of the study protocol have been published elsewhere[12]. In short, all inhabitants of the city of Groningen aged 28–75 years were sent a questionnaire and a vial to collect a first-morning-void urine sample. Of these individuals, 40 856 responded (47.8%). From these individuals a cohort consisting of 8592 subjects was selected (the PREVEND cohort). In this ongoing study participant are invited to visit an outpatient clinic for detailed medical examination at ± 3-year intervals. At each screening round participants fill out questionnaires on demographics, medical history and drug use. Information on drug use is completed with data from community pharmacies, including information on class of antihypertensive medication (ACEi/ARB). At the study visits participants deliver two 24-hour urine collections, blood pressure is measured, anthropometrical measurements are performed, and fasting blood samples are taken.

The PREVEND study was approved by the institutional ethics review board and was conducted in accordance with the guidelines of the Declaration of Helsinki. All participants provided written informed consent.

Definitions

Normoalbuminuria was defined as urinary albumin excretion (UAE) <30 mg/24h, microalbuminuria as UAE 30-299 mg/24h and macroalbuminuria as UAE $\geq$300 mg/24h. Albuminuria status was based on the average of 2 consecutive measurements in 24-hour urine collections. Transition in albuminuria was defined as a transition from normo- to micro- or from micro- to macroalbuminuria with at least 30% increase in urinary albumin excretion from baseline between two consecutive study visits. T2DM was defined as the use of oral anti-diabetic treatment (self-reported or by information retrieved from the regional pharmacy database), a fasting plasma glucose >7.0 mmol/L (126 mg/dL) or

non-fasting plasma glucose >11.1 mmol/L (>200 mg/dL). HT was defined as the use of anti-hypertensive treatment or a systolic/diastolic blood pressure > 140/90 mmHg.

Selection of cases and controls

For the present study, we selected patients with type 2 diabetes mellitus (T2DM) and transition from normo- to microalbuminuria or from micro- to macroalbuminuria. As controls, we selected T2DM patients who had persistent normoalbuminuria or microalbuminuria throughout the same time interval as cases (Figure 1A). As a secondary cohort we selected similar cases and controls out of all non-diabetic patients with hypertension (Figure 1B).

Controls were matched to the cases 1:1 based on diabetes/non-diabetes, age, gender, baseline normo- or microalbuminuria status, and if applicable duration of diabetes. For each subject that showed transition in albuminuria, a matched control was selected that most optimally resembled the case on these combined parameters. Plasma samples of these patients were used from the visit *prior* to the transition from normo- to microalbuminuria, or micro- to macroalbuminuria (baseline). The use of agents intervening in the Renin-Angiotensin-Aldosterone system (RAAS) (ACEi/ARB) was allowed, but the type of drug and their dose had to remain stable during the study period.

Of the 8592 study participants, 318 had T2DM and 33 of these subjects had unambiguous transition in albuminuria and available samples of sufficient quality. Hypertension (without diabetes) was present in 1178 participants. Of these participants 75 subjects had unambiguous transition in albuminuria and available samples of sufficient quality.

Measurements

Plasma samples were stored at -80^{0}C and all samples underwent 1 freeze-thaw cycle. Measurements were performed blinded and in duplicate. GDF15 was measured with a novel pre-commercial assay based on the Eclia principle (Roche Diagnostics) with a LLD of 200 pg/mL and intra-individual CV of 6.7 to 9.2%.

Urinary albumin excretion (UAE) is given as the mean of the two 24-hour urinary excretions. Blood pressure was measured twice, in the supine position, every min for 10 min with an automatic device (Dinamap XLModel 9300; Johnson-Johnson Medical, Tampa, FL, USA). eGFR was estimated using the Modification of Diet in Renal Disease (MDRD) study equation, using gender, age, race and serum creatinine[13].

Urinary albumin concentration was determined by nephelometry (Siemens, Munich, Germany). Concentrations of total cholesterol and plasma glucose were measured using

standard methods. Serum creatinine was measured by dry chemistry (Eastman Kodak, Rochester, New York, USA), with intra-assay coefficient of variation of 0.9% and inter-assay coefficient of variation of 2.9%.

Statistical Analysis

Analyses were performed using STATA version 11.2 (StataCorp LP, Lakeway Drive, Texas, USA). A study with 50 patients will provide at least 80% power to detect an odds ratio of 1.5 assuming a type 1 error of 5% and no residual confounding after matching cases and controls (www.bioconductor.org). Variables with normal distribution are given as mean ± standard deviation and variables with skewed distribution as median [inter quartile range (IQR)]. Variables with skewed distribution were log-transformed for analyses. Graphical methods were used to ascertain normalization of the distribution after transformation. Differences between the cases and controls were tested with paired-samples t-test for continuous variables and chi-squared test on paired proportions for categorical variables. Differences between non-paired groups were tested with independent samples t-test for continuous variables and chi-square test for categorical variables.

To investigate the association between the levels of the marker and transition in albuminuria we used conditional logistic regression because of the paired study design. In multivariable analyses, we adjusted for the differences in baseline for differences in baseline urinary albumin excretion (UAE) and estimated glomerular filtration rate (eGFR) between cases and controls as these two markers are important risk factors for transition in albuminuria stage[14;15]. We tested for interaction between patients who made a transition from normo- to microalbuminuria and those who made a transition from micro- to macroalbuminuria by adding an interaction term for baseline albuminuria status and GDF15 in the model.

To assess whether the markers improved risk prediction and discrimination we determined the c-statistic and Integrated Discrimination Improvement (IDI). We calculated the c-statistic (discriminatory ability) based on the most important established risk factors (UAE, eGFR) and compared those to c-statistics after addition of GDF15 to the established model. Differences in c-statistic were tested with chi-square test.

In addition to the c-statistic, we calculated the integrated discrimination improvement (IDI), another measure of discrimination[16]. The IDI is the difference in discrimination slopes

between cases and controls before and after the addition of the biomarker(s) to the model. It assesses the improvement in average sensitivity without sacrificing average specificity. Calculation of the IDI is done by computing average predicted probabilities of the event in cases and controls in models with and without the biomarker(s) and subtracting the values from cases and controls from each other. The increase in difference between cases and controls after addition of the biomarker(s) is the integrated reclassification improvement. The IDI is often more sensitive than the rather conservative c-statistic because when several highly predictive markers are in the model, enormous odds ratios are required to meaningfully increase the c-statistic[17]. For all analyses two-sided P-values <0.05 were considered statistically significant.

Results

In total, 33 cases with type 2 diabetes mellitus (T2DM) and transition from normo- to microalbuminuria or from micro- to macroalbuminuria were identified. These cases were pair-matched to 33 controls with T2DM and stable albuminuria. The baseline characteristics are presented in Table 1.

Mean age of the patients was 64.9 years, 176 (72%) patients were male, and the median urinary albumin excretion rate was 18 [9-44] g/24h. Cases had a significantly higher baseline UAE than controls, were more frequently treated with lipid lowering treatment and more often received ACEi/ARB treatment when compared to matched controls. All other parameters were similar. Median follow-up time was 2.7 [2.2-4.0] years.

Change in albuminuria concentrations during the follow-up, according to the selection of cases and controls, is shown in Figure 1A. Patients with transition in albuminuria (cases) had a median increase in albuminuria of 31 mg/24h (164%) compared to 0 mg/24h (0%) in controls.

GDF15 in Type 2 Diabetic Patients

The mean concentration of GDF15 in cases and for controls, prior to transition in albuminuria, is presented in Figure 2A. In Figure 2B these concentrations are presented separately for transition from normo- to micro and micro- to macro. GDF15 concentrations were significantly higher in cases vs. controls (1288 [885-1546] vs. 948 [660-1016] pg/mL, P<0.001). The concentrations were lower in normoalbuminuric cases and controls than microalbuminuric cases and controls (910 [737-1162] vs. 1008 [763-1470], P=0.03).

The odd ratio for transition in albuminuria was 3.58 [95%CI 1.51-8.47] (per standard deviation increment in GDF15) (Table 2) and 2.87 [95%CI 1.10-7.53] after adjustment for baseline albuminuria and eGFR. There was no statistical significant difference between the odds for transition from normo- to microalbuminuria and from micro- to macroalbuminuria with each SD increment in GDF15 (P for interaction = 0.55). GDF15 significantly improved the c-statistic on top of a baseline model consisting of UAE and eGFR (increase from 0.87 to 0.92, P=0.03). GDF15 also improved the Integrated Discrimination Index (IDI) (0.148, P=0.001), indicating that GDF15 improved the discrimination between cases and controls beyond baseline UAE and eGFR (Table 3).

Table 1: Baseline Characteristics for patients with T2DM and non-diabetic patients with HT.

	Diabetes		Hypertension	
Patient Characteristic	**Cases**	**Controls**	**Cases**	**Controls**
Number - n	33	33	75	75
Age - years	62.7 ± 9.2	62.8 ± 9.6	66.3 ± 9.8	65.6 ± 8.7
Male gender - n (%)	25 (75.8)	25 (75.8)	53 (70.7)	53 (70.7)
Race - n (%)				
Caucasian	30 (90.9)	31 (93.9)	71 (94.7)	73 (97.3)
Other	3 (9.1)	2 (6.1)	4 (5.3)	2 (2.7)
Smoking - n (%)	6 (18.1)	5 (15.1)	18 (24.0)	13 (17.3)
BMI - kg/m2	30.0 ± 6.3	27.8 ± 4.2	28.5 ± 4.5	27.9 ± 4.5
Systolic Blood Pressure - mmHg	137 ± 15	134 ± 20	139 ± 19	137 ± 18
Diastolic Blood Pressure - mmHg	75 ± 9	75 ± 8	77 ± 8	78 ± 9
History of Coronary Heart Disease[$]- n (%)	7 (21.3)	2 (6.1) †	11 (14.9)	9 (12.0)
Follow-up time	2.7 [2.2-3.8]	2.8 [2.3-4.0]	2.8[2.3-4.0]	2.8 [2.1-3.7]
Laboratory Parameters				
Urinary albumin excretion - mg/24h	22 [17-34]	12 [6-40]*	22 [13-83]	11 [7 -37] ‡
eGFR - mL/min/1.73m2	76 ± 18	80 ± 17	72 ± 21	69 ± 20
Total Cholesterol - mmol/L	5.0 ± 1.4	5.2 ± 1.4	5.3 ± 1.0	5.4 ± 1.1
Fasting Plasma Glucose - mmol/L	7.6 ± 1.9	7.2 ± 1.2	5.3 ± 0.8	5.1 ± 0.9
Treatment - n (%)				
Antihypertensive drugs	23 (69.7)	11 (33.3)	75 (100)	75 (100)
ACEi/ ARB	16 (48.5)	2 (6.1)*	39 (52.0)	39 (52.0)
Oral antidiabetics	24 (72.7)	24 (72.7)	0 (0)	0 (0)
Lipid lowering drugs	18 (54.6)	7 (21.2) †	27 (36.0)	20 (26.7)
Baseline UAE status - n (%)				
Normoalbuminuria	24 (72.7)	24 (72.7)	50 (66.7)	50 (66.7)
Microalbuminuria	9 (27.3)	9 (27.3)	25 (33.3)	25 (33.3)
Median change in UAE - mg/24h	31 [22-151]	0 [-4 - 3] ‡	70 [29-310]	0 [-2 – 8] ‡
Median change in UAE - %	164 [106-340]	0 [-23 – 38]	314 [143-668]	5 [-18 – 46]
Baseline Biomarker Levels				
GDF15 - pg/mL	1288 [885-1546]	948 [660-1016]‡	975 [739-1222]	872 [726-1172]

*Cases vs. controls P < 0.05, † Cases vs. controls P<0.01, ‡ Cases vs. controls P<0.001. Plus-minus values are means ± SD and non-normally distributed variables are median [inter quartile range (IQR)]. $ Self-reported Coronary Heart Disease. T2DM: Type 2 Diabetes Mellitus; HT: Hypertension; BMI: body mass index (weight kg/length m^2); eGFR: estimated glomerular filtration rate (modification of diet in renal disease-formula); ACEi: angiotensin-converting enzyme inhibitors; ARB: angiotensin-2 receptor blockers; UAE: urinary albumin excretion rate. To convert values for serum cholesterol from mmol/L into mg/dL multiply by 38.67, to convert values for fasting plasma glucose from mmol/L into mg/dL multiply by 18 and to convert values for serum creatinine from µmol/L to mg/dL divide by 88.4.

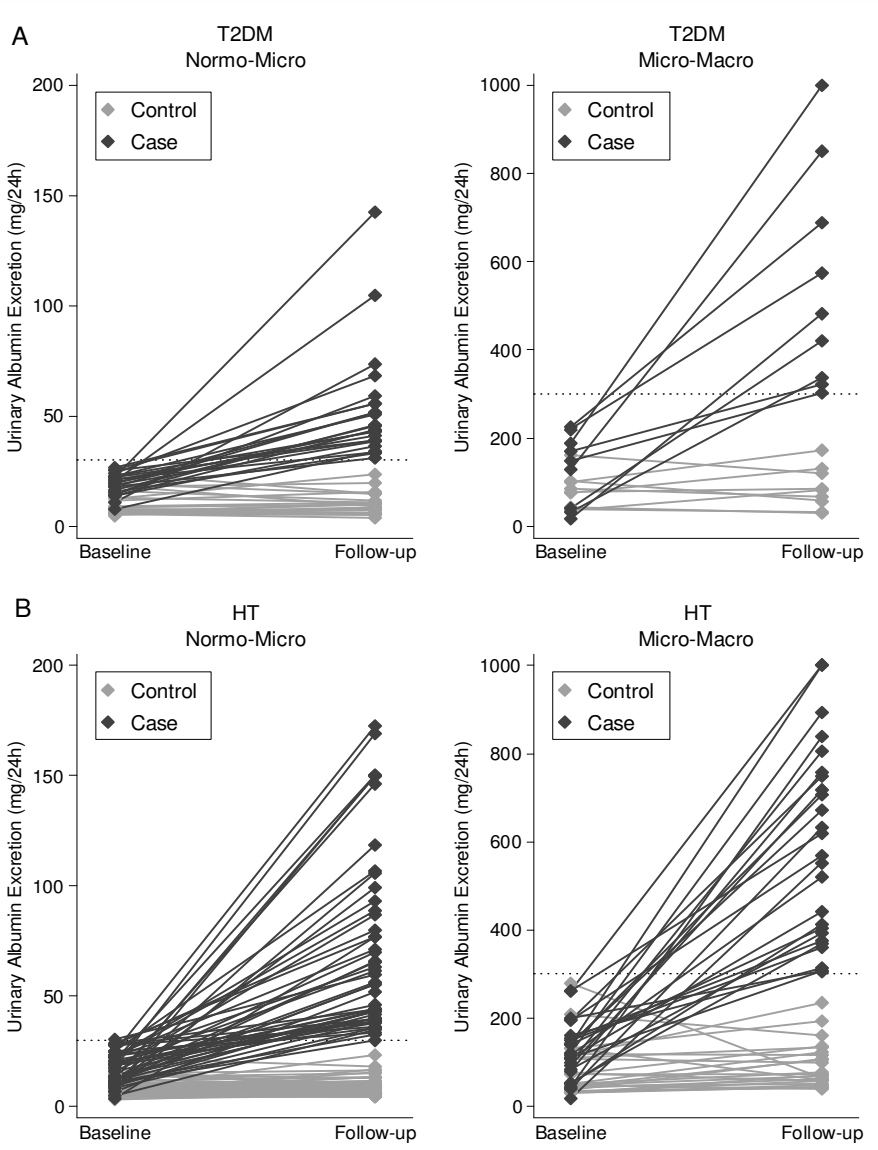

Figure 1. Individual courses of urinary albumin excretion in progressors and non-progressors in albuminuria (cases and controls). Individual course of UAE during follow-up for cases (black) and controls (grey) in patients with diabetes (1A) or hypertension (1B) stratified for albuminuria-status at baseline (left: normoalbuminuria; right: microalbuminuria).

GDF15 in non-diabetic Hypertensive patients

The replication study was a second nested case-control study, which consisted of 75 (non-diabetic) cases with hypertension (HT) and transition to increasing stages of albuminuria that were pair-matched to 75 controls with HT and stable albuminuria (Table 1). Characteristics of patients with HT were remarkably similar to patients with T2DM, as was the median follow-up time (2.8 [2.3-3.9] years in HT vs. 2.7 [2.2-4.0] years in T2DM). Cases with HT had a median increase in albuminuria of 70 mg/24h (314%) compared to 0.2 mg/24h (5%) in controls.

The GDF15 concentration in patients with HT was similar to the concentration in patients with T2DM (910 [738-1210] vs. 992 [799-1347] resp.; P=0.63). In cases with HT GDF15 concentration was borderline significantly higher than in controls (975 [739-1222] vs. 872 [726-1172] pg/mL, P=0.09). The odds of transition to micro- or macroalbuminuria was significantly increased per SD increment in GDF15 (1.96 [95%CI 1.12-3.45]). Also in patients with HT no difference was observed in the odds for transition from normo- to microalbuminuria or micro- to macroalbuminuria with each increment in GDF15 (p for interaction = 0.90). Addition of GDF15 to the prediction model of transition in albuminuria, consisting of baseline UAE and eGFR, did not result in significant improvement of the c-statistic (from 0.88 to 0.89, P=0.8), but significantly improved IDI (0.059, P=0.04).

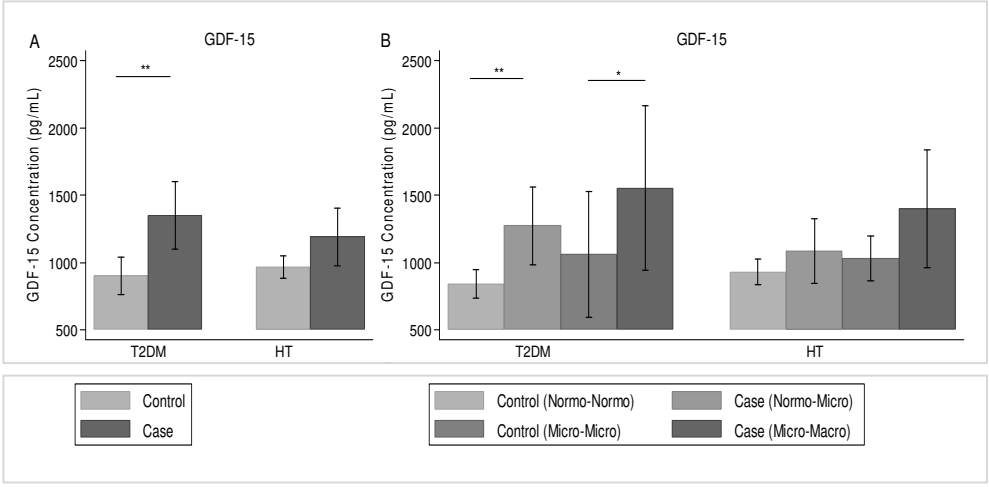

Figure 2. GDF15 levels in progressors and non-progressors in albuminuria (cases and controls). GDF15 levels (pg/mL) (mean ± SD) in progressors and non-progressors in albuminuria for T2DM and hypertension; 2B: GDF15 levels (pg/mL) (mean ± SD) in progressors and non-progressors in albuminuria for T2DM and hypertension stratified for baseline albuminuria (normoalbuminuria or microalbuminuria); * P<0.05, ** P<0.001

Table 2: Odds ratios for GDF15 and transition in albuminuria per log-unit increase and per SD increase in the level of the biomarker for T2DM and non-diabetic patients with hypertension.

Type 2 Diabetes Mellitus			Per doubling		Per SD increase	
Biomarker		**Odds ratio**	**95% Conf. Int.**	**Odds ratio**	**95% Conf. Int.**	***P*-value**
GDF15	Crude	7.66	[1.94-30.14]	3.58	[1.51-8.47]	0.004
GDF15	Adjusted *	5.39	[1.16-25.05]	2.87	[1.10-7.53]	0.03
Hypertension						
Biomarker		**Odds ratio**	**95% Conf. Int.**	**Odds ratio**	**95% Conf. Int.**	***P*-value**
GDF15	Crude	1.58	[0.92-2.73]	1.33	[0.95-1.88]	0.10
GDF15	Adjusted	2.94	[1.20-7.20]	1.96	[1.12-3.45]	0.02

* Adjusted for baseline albuminuria and estimated glomerular filtration rate (eGFR)
Values were calculated with conditional logistic regression analyses

Table 3: C-statistics and integrated discrimination improvements (IDI) for logistic regression models predicting transition in albuminuria in patients with type 2 diabetes mellitus and non-diabetic patients with hypertension.

Type 2 Diabetes Mellitus	C-statistic	95% Conf.Int	*P*-value	IDI	95% Conf.Int	*P*-value
Established Model*	0.87	[0.78-0.96]	*reference*	*reference*		*Reference*
Established Model + GDF15	0.92	[0.86-0.99]	0.03	0.148	[0.056-0.240]	0.001
Hypertension						
Established Model*	0.88	[0.82-0.94]	*reference*	*reference*		*Reference*
Established Model + GDF15	0.89	[0.83-0.94]	0.8	0.045	[0.006-0.083]	0.02

* Established model: prediction model based on urinary albumin excretion and estimated glomerular filtration rate. The biomarker was modeled as continuous (log-transformed) variable. P values are for comparison of the models with the baseline model.

Discussion

To our best of knowledge, this is the first study to indicate that GDF15 precedes and predicts the development of micro- or macroalbuminuria in patients with type 2 diabetes mellitus. GDF15 was not only independently associated with transition in albuminuria during a follow-up of ~3 years, it also improved discrimination between patients who did and did not develop micro- or macroalbuminuria, beyond conventional risk markers. These findings were extended and replicated in a cohort of non-diabetic hypertensive patients.

Previous studies mainly implicated GDF15 as predictor for cardiovascular events and all-cause mortality in patients with previous myocardial infarction[11;18;19]. The only previous study focusing on GDF15 and renal outcome was performed by Lajer et al in patients with type I diabetes mellitus and macroalbuminuria. In this observational study, higher GDF15 levels were associated with a faster decline of eGFR and development of end-stage renal disease[11]. Our study extends these latter findings to earlier stages of renal disease during which appropriate intervention is most beneficial.

Despite a growing body of literature demonstrating that GDF15 is a valuable biomarker for cardiovascular disease, relatively little is known on the pathophysiological role of GDF15. Its expression is markedly increased in response to injury in various tissues, including heart and kidney, and is believed to be a protective factor by reducing apoptosis and influencing cellular proliferation[20-22]. Higher levels of GDF15 may thus indicate tissue damage. As endothelial cells appear to be a prominent source of GDF15, the circulating levels of GDF15 most likely represent generalized endothelial and microvascular damage[23]. The relation of GDF15 with microvasculature may explain the link with albuminuria, as albuminuria supposedly reflects established microvascular damage in the renal and peripheral vasculature[24]. Since our cases and controls were at the same albuminuria (either normo- or microalbuminuria) at time of GDF15 measurement, GDF15 seems to already be increased before microalbuminuria becomes detectable. Our finding that GDF15 also contributed in identifying subjects at risk for transition in albuminuria stage in a non-diabetic hypertensive cohort implies that GDF15 is not specifically related to diabetes. Future research will be necessary to clear up the exact pathophysiological role of GDF15 in diabetes or hypertension and to assess its relation with albuminuria.

Traditional risk factors associated with transition in albuminuria are age, gender, dyslipidemia, hypercholesterolemia, insulin sensitivity, hyperglycemia, duration of

diabetes, increased blood pressure, duration of hypertension, body mass index and smoking[7;25]. Despite identification of these important risk factors for transition in albuminuria, the ability to stratify subjects at risk is still limited. The current data, if confirmed, raise the possibility of identification of subjects at risk even when traditional risk factors do not indicate risk, facilitating early identification of those with an increased chance of developing micro- or macro- albuminuria. Because early intervention is more effective than late intervention, preventive treatment could thus reduce the risk of renal and cardiovascular events in patients with T2DM[5;6]. Our findings also applied to non-diabetic hypertensive patients, in whom albuminuria is strongly associated with renal and cardiovascular events[8;26].

We incorporated prediction analyses in the present study to determine GDF15 could add to risk prediction in individual subjects beyond traditional risk markers. Whereas the c-static is most commonly used, the c-statistic is not very sensitive in detecting small but meaningful contributions of biomarkers in correctly classifying individuals. Hardly any improvement of the c-statistic can be reached when the model already includes one or several important risk markers[17]. Yet, we found improvement in c-statistic in our study in T2DM. Because of the insensitivity of the c-statistic, other measures of risk classification such as the IDI have recently been proposed[16;28]. In our analyses, we also found significant improvement in IDI for GDF15, both in patients with T2DM and non-diabetic subjects with hypertension.

The availability of patient samples from the large general population cohort PREVEND was a unique feature of this study. Because this study followed the natural course of albuminuria during by performing repeated measurements of albuminuria for more than 10 years, we were able to measure GDF15 in samples prior to the transition in albuminuria, whereas many study have a cross-sectional design and test biomarkers in patients with established increased albuminuria.

Other strengths include the well-defined phenotype of the population, with albuminuria status based on two consecutive 24-hour urine collections, the rigorous definitions for transition in albuminuria, and the availability of samples that had never been thawed. Because of these strict criteria and the limited number of patients with diabetes in this general population cohort we were only able to obtain 33 valid cases.

A limitation of this study was the modest difference in baseline albuminuria. This small baseline difference in albuminuria was inherent to the design of the study, as the level of albuminuria in itself is related to transition to increase stages of albuminuria and we matched by albuminuria-stage rather than by albuminuria level. Adjustment for baseline albuminuria showed that the association of GDF15 with albuminuria transition was independent of baseline albuminuria.

A second limitation is the fact that the nested case-control design of the study may overestimate the true predictive capacity of the models because of the relative high event rate in case-control studies[29]. Absolute values of the c-statistic should therefore be interpreted with caution. The improvement of the c-statistic and the level of significance, however are unaffected by case-control design of studies. Lastly, whereas these results are promising and could be confirmed in non-diabetic hypertensive patients, replication of the current results in other studies, preferably large prospective cohort studies, is warranted before final conclusions can be reached.

In conclusion, we identified GDF15 as marker for prediction of transition in albuminuria in type 2 diabetic subjects. GDF15 moreover had significant additive value on top conventional risk markers in the prediction of albuminuria-transition. These finding were confirmed in non-diabetic hypertensive subjects. If these findings prove to be replicable in other studies, GDF15 might be a valuable marker for individual risk stratification, facilitating start or intensification of treatment in high risk patients in order to prevent or delay the progression of nephropathy.

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Chapter 7

Summary, discussion and future perspectives

Summary

Type 2 diabetes mellitus (T2DM) is the most common type of diabetes and the prevalence of T2DM is increasing rapidly due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. The incidence of type 1 diabetes mellitus (T1DM) is also increasing although the underlying mechanisms are less well understood. Diabetic nephropathy is a common complication of diabetes and cause of chronic renal failure, resulting in an increased number of patients requiring end-stage renal failure management, particularly dialysis[1]. Therefore, new therapies to prevent or halt the progression of diabetic nephropathy are urgently needed[2].

Prevention of fibrosis has been identified as a possible target for delaying the progression of diabetic nephropathy. One of the most prominent and most frequently studied cytokine involved in these processes is Transforming Growth Factor beta (TGF-β). TGF-β is part of an extensive family of signaling molecules including the three different isoforms of TGF-β, activins, inhibins, growth differentiation factors (GDFs), and bone morphogenetic proteins (BMPs)[3].

Despite the overwhelming body of literature on the involvement of TGF-β in the development of diabetic nephropathy, we hypothesized that other growth factors may be involved in the development of diabetic nephropathy. Furthermore, the involvement of these factors may have been obscured by the fact that most experimental studies investigating the development of diabetic nephropathy were conducted several weeks after the induction of experimental diabetes. We therefore set out to identify new factors involved in the induction of diabetic nephropathy in the first week of experimental diabetes using an expression micro-array experiment. In *Chapter 2* we identified several genes which were associated with the induction of renal fibrosis in diabetes. Our micro-array analysis was the first to show that Growth Differentiation Factor 15 (GDF15) was upregulated in the kidney immediately after induction of experimental diabetes. Moreover, we confirmed GDF15 gene expression by RT-PCR and western blot. The upregulation of GDF15 was particularly present in the glomerulus and preceded changes in TGF-β expression. These results led us to hypothesize that GDF15 is a factor involved in the development of diabetic nephropathy.

Consequently, we aimed at establishing the role of GDF15 in development of diabetic nephropathy in an *in vitro* model. As GDF15 expression was highly induced in the

glomerulus and because mesangial cells are the prime cell type involved in excess glomerular extracellular matrix production, we investigated the functional effects and signaling pathways of GDF15 in cultured mesangial cells (*Chapter 3*). GDF15 did not affect fibrosis and apoptosis but stimulated proliferation of human mesangial cells (THMC) through activation of the MAPK members JNK and p38 and involved the BMPR2 receptor. In contrast, unlike TGF-β, the signaling evoked by GDF15 in THMC did not employ the classical Smad pathway.

Although *Chapter 3* demonstrated the receptor and signaling cascades of GDF15 in a renal cell type, it did not fully clarify the role for GDF15 in diabetic nephropathy. To further investigate the role of GDF15 in diabetes, we evaluated the development of diabetic nephropathy in GDF15 knockout mice and wild type mice in models of type 1 and type 2 diabetes (*Chapter 4*). Genetic deletion of GDF15 resulted in augmented tubular and interstitial damage in both models of diabetes, despite a similar diabetic state in GDF15 knockout and wild type mice. Increased tubular damage in knockout animals was associated with increased glucosuria and polyuria in both models. While diabetic knockout mice showed increased interstitial damage, glomerular damage was similarly elevated in diabetic knockout and wild type mice. In the type 1 model, GDF15 knockout mice demonstrated increased expression of inflammatory markers. In the type 2 model, elevated levels of plasma creatinine indicated impaired kidney function in knockout mice. Therefore, this chapter demonstrated that GDF15 protected the kidney from diabetic damage and maintained renal tubular function in both type 1 and type 2 models of diabetes.

Cardiovascular disease (CVD) and development of diabetic nephropathy are closely connected. Impairment of the endothelium, the cells lining the blood vessels, results in vascular dysfunction in diverse vascular beds including the kidney. In addition, endothelial dysfunction causes increased protein leakage over the filtration barrier of the kidney, resulting in albuminuria. Both vascular dysfunction and albuminuria contribute to the progression of diabetic nephropathy.
As GDF15 is emerging as a biomarker for CVD, we investigated the effects of GDF15 on vascular and endothelial function. In *Chapter 5*, we demonstrated that GDF15 played an important role in the inhibition of vascular contraction and relaxation in both wild type and GDF15 knockout mice. Phenylephrine induced contractions and Acetylcholine (ACh) mediated relaxations were similar in wild type and GDF15 knockout mice. Pretreatment with GDF15 inhibited contraction in both wild type and knockout groups by increasing

basal nitrous-oxide (NO) release. Pretreatment with GDF15 also inhibited ACh induced relaxation in both wild type and knockout groups by reducing the stimulated NO release. Disruption of caveolae resulted in a similar inhibition of vascular responses as GDF15 and administration of GDF15 with the caveolae disrupting agent methyl-β-cyclodextrin (MCD) did not result in a further inhibition of vascular responses. Therefore, GDF15 impaired endothelial function by modulating endothelial NO-synthase activity, possibly through a mechanism that involved modification of caveolar signaling of the endothelium.

To further explore the relationship between GDF15 and renal endothelial dysfunction, we aimed at establishing the relationship between GDF15 and progression of albuminuria in humans (*Chapter 6*). For this, we carefully selected cases and controls. Cases were defined by transition from normo- to micro-, and micro- to macro-albuminuria. Controls with stable albuminuria were pair-matched for age, gender, albuminuria-status and diabetes-duration. Two different populations were studied: T2DM patients and patients with hypertension. Plasma GDF15 levels were measured in both populations at baseline, i.e. prior to the transition in the extent of albuminuria. Baseline GDF15 levels prior to transition were higher in cases than in controls. Moreover, GDF15 was independently associated with progression in albuminuria in T2DM and improved the prediction of progression of albuminuria with a higher c-statistic and Integrated Discrimination Improvement (IDI). Furthermore, these finding were extended to non-diabetic subjects with hypertension. Therese results demonstrate that baseline GDF15 level may represent a valuable prognostic marker for the progression of albuminuria and renal endothelial dysfunction.

In summary, the studies presented in this thesis demonstrated a profound role for GDF15 in the development of diabetic nephropathy. GDF15 expression was strongly induced in the glomerulus after induction of diabetes in rats and mice. Further, our study in GDF15 knockout and wild type mice showed that GDF15 preserved tubular function and prevented interstitial damage in both type 1 and type 2 diabetes. However, GDF15 did not protect the glomerulus from sclerosis nor did it prevent albuminuria. Furthermore, pretreatment of vessels (aortas) with GDF15 affected endothelial function resulting in impaired vascular contraction and relaxation. Finally, plasma levels of GDF15 correlated with progression of albuminuria in patients with T2DM. The notion that BMPR2 seems to be involved as a main receptor for GDF15, may facilitate development of novel therapeutic approaches to limit renal damage in diabetic patients.

Discussion and future perspectives

In our studies, GDF15 was found to protect the tubulus and renal interstitium from development of diabetic damage. The protective effects of GDF15 have previously been demonstrated in the heart and cultured cardiomyocytes after induction of myocardial infarction[4;5] and ischemia/ reperfusion[6]. In addition, the protective effects of GDF15 have been suggested in several other organs and tissues in response to diverse noxious stimuli, including surgical, toxic, ischemic, and hyperoxic damage to lung and kidney[7], surgical and chemical injury and heat shock to liver[8] and ischemia of the bile duct[9].

The mechanisms through which GDF15 protects these tissues and organs are diverse. Inhibition of apoptosis by GDF15 has been suggested in endothelium, heart, lung and kidney[4;6;10-12]. In addition, GDF15 affects cellular proliferation in lung and kidney[10;13], suggesting that GDF15 may be involved in regeneration of specific cell types after injury. In the kidney, GDF15 mediated regeneration of epithelial cells in mouse kidney collecting duct in response to metabolic acidosis[13]. Finally, GDF15 may inhibit inflammation as GDF15 was found to modulate leukocyte integrin activation on the endothelium in the heart[14]. In our studies in models of diabetic nephropathy, we confirmed the effects of GDF15 on proliferation and inflammation, although we did not find effects of GDF15 on apoptosis.

Despite the protective properties of GDF15 in several organs and tissues, several studies reported a correlation between plasma GDF15 levels and mortality and morbidity in CVD[15-20] and in diabetes[21-23]. Furthermore, our study in T2DM patients demonstrated that increased levels of GDF15 predicted the progression of albuminuria in diabetes and hypertension (*Chapter 6*).

These apparent opposing effects of GDF15 may be explained by the findings described in this thesis.

First, in *Chapter 2* and *3* we demonstrated that the glomerulus is a source of GDF15 immediately after induction of diabetes in rats and mice. Furthermore, *Chapter 6* demonstrated that plasma GDF15 levels predicted the progression of glomerular diabetic damage in patients with T2DM. Therefore, plasma GDF15 levels appear to reflect early glomerular damage in diabetes. Further, in *Chapter 4* we demonstrated that the interstitium and tubules were the main targets for GDF15 in the diabetic kidney. As genetic deletion of GDF15 did not affect diabetic glomerular damage, these data indicate that the source and

targets of GDF15 are distinct. Therefore plasma GDF15 levels may reflect the extent of diabetic glomerular damage (which is not affected by GDF15), while GDF15 provides protection to downstream tissues such as the tubulus and renal interstitium.

Secondly, in *Chapter 5* we demonstrated that GDF15 had profound effects on vascular function as GDF15 treatment of isolated aortic rings resulted in inhibited vascular contraction and relaxation by modulating the function of the endothelium. The impaired relaxation mediated by GDF15 resembled endothelial dysfunction observed in several models of CVD, an established determinant of susceptibility to end-organ damage[24-29]. Therefore these results suggest that while GDF15 provides protection to injured organs and tissues, long term elevation of GDF15 levels may result in endothelial dysfunction contributing to the development of CVD and kidney disease.

In conclusion, the data presented in this thesis demonstrates a profound role for GDF15 in the development of diabetic nephropathy. GDF15 protects the tubulus and renal interstitium in diabetes and is a prognostic marker for the progression of albuminuria.

In future experiments, we aim at development of kidney specific GDF15 knockout mice to further elucidate the role of renal GDF15 production in diabetes. The role of the BMPR2 as GDF15 receptor in mediating GDF15 effects will be investigated in other renal tissues, including the tubulus. Furthermore, the identification of the GDF15 receptor provides new tools for intervention into the GDF15 signaling system, allowing to the development of new therapeutic strategies to treat diabetic nephropathy.

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Nederlandse samenvatting

Samenvatting

Ouderdomsdiabetes of type 2 diabetes mellitus (T2DM) is de meest voorkomende vorm van diabetes en het aantal patiënten met T2DM neemt snel toe als gevolg van bevolkingsgroei, vergrijzing, verstedelijking en door toename van obesitas en lichamelijke inactiviteit. Het aantal gevallen van juveniele of type 1 diabetes mellitus (T1DM) neemt ook toe, hoewel de onderliggende mechanismen minder goed bekend zijn. Een veel voorkomende complicatie van diabetes is schade aan de nieren, een aandoening die bekend staat als diabetische nefropathie. Diabetische nefropathie is een belangrijke oorzaak voor het ontstaan van chronisch nierfalen, resulterend in een toename in het aantal patiënten met eindstadium nierfalen die afhankelijk is van nier dialyse. Gezien de verwachte toename in het aantal diabetes patienten, zijn er dringend nieuwe therapieën nodig om diabetische nefropathie te voorkomen, of de progressie ervan te stoppen.

Diabetische nefropathie wordt onder andere gekenmerkt door verhoogde productie van bindweefsel in de nier (fibrose) en preventie van fibrose is een mogelijk doelwit voor het vertragen van de progressie van diabetische nefropathie. Een van de meest prominente en meest bestudeerde eiwitten die betrokken zijn bij het fibrose proces is de cytokine Transforming Growth Factor beta (TGF-β). TGF-β is een onderdeel van een uitgebreide familie van signaalmoleculen, waartoe drie verschillende vormen van TGF-β, activins, inhibins, growth differentiation factors (GDFs), en bone morphogenic proteins (BMP) behoren. Ondanks de overweldigende hoeveelheid literatuur over de betrokkenheid van TGF-β in het ontstaan van nierfibrose, veronderstelden wij dat ook andere groeifactoren betrokken zijn bij het ontstaan van nierfibrose ten gevolge van diabetes. De betrokkenheid van deze factoren zijn mogelijk onopgemerkt gebleven doordat de meeste studies naar de ontwikkeling van diabetische nefropathie in proefdieren pas relatief laat na het induceren van diabetes werden uitgevoerd.

Onze eerste studie was daarom gericht op het identificeren van nieuwe factoren die betrokken zijn bij het ontstaan van nierfibrose in de eerste week van de experimentele diabetes met behulp van een expressie micro-array experiment. In *Hoofdstuk 2* identificeerden wij genen die geassocieerd waren met de inductie van nierfibrose in diabetische ratten. Onze micro-array analyse toonde als eerste aan dat de genexpressie van Growth Differentiation Factor 15 (GDF15) sterk verhoogd was in de nier onmiddellijk na inductie van experimentele diabetes. De verhoogde expressie van GDF15 was met name aanwezig in de glomerulus en ging vooraf aan veranderingen in TGF-β expressie. Deze

resultaten leidden ons tot de hypothese dat GDF15 een factor is die betrokken is bij de ontwikkeling van nierfibrose en diabetische nefropathie.

Om onze hypothese te toetsen, werd het effect van GDF15 op fibrose onderzocht met behulp van in-vitro modellen. Omdat GDF15 met name tot expressie kwam in de glomerulus, hebben wij de effecten van GDF15 onderzocht op het type cellen die betrokken zijn bij fibrose van de glomerulus, de zogenaamde mesangiale cellen (*Hoofdstuk 3*). GDF15 had geen invloed op fibrose en apoptose, maar stimuleerde proliferatie van gekweekte mesangiale cellen door middel van activatie van de BMPR2 receptor en de MAPK signaalcascade. GDF-15 activeerde niet de klassieke TGF-β signaalcascades.

Om de rol van GDF15 in diabetes verder te onderzoeken, hebben we vervolgens gebruik gemaakt van muizen waarin het GDF15 gen verwijderd was, zogenaamde GDF15 knockout muizen. In *Hoofdstuk 4* werd de ontwikkeling van diabetische nefropathie vergeleken tussen GDF15 knockout muizen en wild-type muizen in modellen van type 1 en type 2 diabetes. Genetische deletie van GDF15 resulteerde in verhoogde nier schade met name aan de tubuli en het interstitium in beide diabetische modellen. Bovendien hadden GDF15 knockout dieren meer glucose verlies in de urine en verhoogde urineproductie. Schade aan de glomerulus was echter vergelijkbaar in GDF15 en wild type muizen. Daarmee is in dit hoofdstuk aangetoond dat GDF15 de nieren beschermd tegen diabetische schade aan tubulus en interstitium en dat GDF15 belangrijk is voor het instant houden van tubulus functie in zowel type 1 en type 2 modellen van diabetes.

Hart- en vaatziekten en de ontwikkeling van diabetische nefropathie zijn nauw met elkaar verbonden. Aantasting van het endotheel, de epitheelcellen van de bloedvaten, resulteert in een vasculaire dysfunctie in diverse vaatsystemen waaronder die van de nieren. Endotheliale dysfunctie veroorzaakt daarnaast een verhoogde lekkage van eiwit over het filtratiemembraan van de nier, wat resulteert in albuminurie. Zowel vasculaire dysfunctie als albuminurie dragen bij aan de progressie van diabetische nefropathie. Omdat GDF15 in opkomst is als een biomarker voor hart-en vaatziekten, hebben we de effecten van GDF15 onderzocht op vasculaire en endotheliale functie. *Hoofdstuk 5* toont aan dat GDF15 een belangrijke rol speelt in de vermindering van vasculaire contractie en relaxatie in zowel wild-type en GDF15 knock-out muizen. Fenylefrine geïnduceerde contractie en acetylcholine (ACh) gemedieerde relaxatie was gelijk in wild type en GDF15 knock-out muizen. Voorbehandeling met GDF15 verminderde de contractie in zowel wildtype als

knockout groepen door een verhoogde basale stikstofmonoxide (NO) productie. GDF15 verminderde daarentegen ACh geïnduceerde relaxatie in zowel wildtype en de knock-out groepen door het verminderen van de gestimuleerde afgifte van NO. Disruptie van caveolae resulteerde in een soortgelijke remming van vasculaire functie als voorbehandeling met GDF15 en toediening van GDF15 met MCD resulteerde niet in een verdere remming van de vaatfunctie. GDF15 verslechtert dus de endotheelfunctie door het moduleren van endotheliale NO-synthase middels een mechanisme van veranderde caveolaire signaaltransductie.

Om de relatie tussen GDF15 en renale endotheliale dysfunctie verder te onderzoeken, hebben we ons gericht op de relatie tussen GDF15 spiegels in het bloed en de progressie van eiwitlek in de urine (albuminurie) bij mensen (*Hoofdstuk 6*). GDF15 spiegels werden hiertoe vergelijken tussen patiënten met progressieve albuminurie ('cases') en patiënten met stabiele albuminurie ('controles'). De eigenschappen van de patiënten in de controle groep waren gelijk aan die van de 'cases' groep voor leeftijd, geslacht, albuminuriestatus en diabetesduur. Twee verschillende populaties werden onderzocht: T2DM patiënten en patiënten met hypertensie. Plasma GDF15 niveaus werden voorafgaand gemeten in beide populaties, dat wil zeggen vóór de progressie in albuminurie. Baseline GDF15 waardes van vóór de progressie waren hoger in de 'cases' groep dan in de controlegroep. GDF15 was onafhankelijk geassocieerd met progressie in albuminurie in T2DM en verbeterde de voorspelling van de progressie van albuminurie met c-statistiek en geïntegreerde discriminatie verbetering (IDI). Deze resultaten tonen aan dat het baseline GDF15 niveau mogelijk een belangrijke prognostische marker is voor de progressie van albuminurie en renale endotheliale dysfunctie, met name in patiënten met T2DM.

Sammenvattend, de data gepresenteerd in dit proefschrift demonstreren een grote rol voor GDF15 in de ontwikkeling van diabetische nefropathie. GDF15 expressie was sterk geïnduceerd in de glomerulus na inductie van diabetes bij ratten en muizen. Verder toonde onze studie in GDF15 knockout en wild type muizen aan dat GDF15 tubulaire functie in stand houdt en interstitiële schade voorkomt in zowel type 1 en type 2 diabetes. Echter, GDF15 beschermde de glomerulus niet tegen sclerose en beschermde niet tegen albuminurie. Bovendien tastte voorbehandeling van aorta's met GDF15 de endotheelfunctie aan, met als gevolg een verstoorde vasculaire contractie en relaxatie. Ten slotte zijn plasmaspiegels van GDF15 gecorreleerd aan de progressie van albuminurie bij patiënten met T2DM. De vinding dat BMPR2 lijkt te zijn betrokken als één van de belangrijkste receptoren voor GDF15, kan de ontwikkeling van nieuwe therapeutische benaderingen voor het beperken van nierschade bij diabetespatiënten bespoedigen.

Slovak summary

Záverečná diskusia a možná implementácia pre prax

Záver a možná implementácia pre prax

Diabetes mellitus 2 typu (T2DM) sa v porovnaní s prvým typom vyskytuje častejšie a jeho prevalencia sa alarmujúco zvyšuje v dôsledku rastu počtu obyvateľstva, starnutia populácie, urbanizácie, rastúceho výskytu obezity a nedostatku telesnej aktivity.

Jednou z najzávažnejších komplikácií diabetu a hlavnou príčinou chronického zlyhania funkcie obličiek je diabetická nefropatia. Vedie k zvyšujúcemu sa počtu pacientov, u ktorých je potrebná liečba pokročilého štádia poruchy funkcie obličiek, až ich zlyhania, pričom často vedie až k dialýze. Preto je nevyhnutne potrebný rozvoj inovačných terapeutických prístupov, zameraných na prevenciu, či zastavenie progresie diabetickej nefropatie.

Jedným z cieľom oddialenia progresie diabetickej nefropatie je stanovenie rozsahu fibrózy. Transformujúci rastový faktor beta (Transforming Growth Factor beta-TGF-β) patrí medzi najvýznamnejšie a najviac študované cytokíny zapojené do fibrotického procesu. TGF-β je súčasťou rozsiahlej rodiny signálnych molekúl, kde patria aj rôzne jeho izoformy, ako aktivíny, inhibíny, rastové diferenciačné faktory (Growth Differentiation Factors-GDFs), a kostné morfogenetické proteíny (Bone Morphogenetic Protein-BMP).
Na základe veľkého množstva literatúry opisujúcej účasť TGF-β vo vývoji diabetickej nefropatie predpokladáme, že na jej rozvoji sa môžu podieľať aj ďalšie rastové faktory z tejto rodiny.

Väčšina štúdií, zameraných na vývoj diabetickej nefropatie, prebieha až niekoľko týždňov po vyvolaní diabetu. Preto sme sa s pomocou DNA microarray rozhodli vyhľadať nové faktory podieľajúce sa na vzniku diabetickej nefropatie už v prvom týždni po vyvolaní epxerimentálneho diabetu. V *kapitole 2* sme identifikovali niekoľko génov, ktoré boli spojené s indukciou renálnej fibrózy u diabetických potkanov. Ukázali sme po prvý krát, že rastový diferenciačný faktor 15 (Growth Differentiation Factor 15-GDF15) je bezprostredne po vyvolaní experimentálneho diabetu 1. typu v obličkách zvýšený. Naviac sme jeho zvýšenú expresiu potvrdili aj pomocou metód RT PCR a Western blotu. Zvýšená expresia GDF15 bola obzvlášť viditelná v glomerulách. Tieto výsledky viedli k predpokladu, že práve GDF15 je faktor, ktorý sa podieľa na rozvoji diabetickej nefropatie.

Na základe tohoto zistenia sme sa zamerali na skúmanie úlohy GDF15 vo vývoji diabetickej nefropatie *v in vitro* modeli. Pretože expresia GDF15 je zvýšená v glomerulách

a primárny typ buniek podieľajúcich sa na vzniku glomerulárneho extracelulárneho matrixu sú hlavne mezangiálne bunky, skúmali sme účinok a signálne dráhy GDF15 v kultivovaných mezangiálnych bunkách (*kapitola 3*). I keď GDF15 v uvedenom *in vitro* modeli neovplyvnil fibrózu ani apoptózu, stimuloval proliferáciu ľudských mezangiálnych buniek (THMC), a to cez aktiváciu MAPK členov JNK a p38, prostredníctvom BMPR2 receptorov. Na rozdiel od TGF-β, intracelulárne signály vyvolané GDF15 v THMC nepôsobia prostredníctvom klasickej Smad kaskády.

I keď *kapitola 3* poukazuje na existenciu receptorov a signálnych kaskád GDF15 v mezangiálnych bunkách obličiek, jeho úloha v diabetickej nefropatii zostáva neodhalená. Pre objasnenie úlohy GDF15 pri diabete sme sledovali vývoj diabetickej nefropatie u knockout myší (KO) bez GDF15 a „divokých" (wild type) myší ako v modeloch prvého, tak aj druhého typu diabetu (*kapitola 4*). Genetické odstránenie GDF15 vyústilo do zvýšeného poškodenia v tubuloch a interstíciu v oboch modeloch diabetu, a to aj napriek podobnému štádiu diabetu u GDF15 knockout myšiach a wild type myšiach. Poškodenie tubulov bolo sprevádzané zvýšenou glykozúriou i polyúriou v oboch modeloch. Kým diabetické knockout myši vykazovali zvýšené poškodenie interstícia, vplyv na glomerulárnu funkciu u diabetických knockout myší a divokých myší bol nezmenený. V modeli s diabetom prvého typu GDF15 knockout myši preukázali zvýšenú expresiu zápalových bio markerov. V modeli diabetu typu 2 sme evidovali zvýšenú hladinu kreatinínu v plazme. V tejto kapitole sme teda ukázali, že GDF15 chráni obličky pri diabetickom poškodení a vplýva na udržaní renálnej tubulárnej funkcie v oboch typoch diabetu.

Kardiovaskulárne ochorenia (KVO) a rozvoj diabetickej nefropatie sú úzko spojené. Poškodenie funkcie endotelu vedie k cievnej dysfunkcii v rôznych typoch ciev. Endotelová dysfunkcia spôsobuje zvýšený únik proteínov cez filtračné membrány obličiek, čo vedie k albuminúrii. Cievna dysfunkcia ako aj albuminúria prispievajú k progresii diabetickej nefropatie.

Pretože GDF15 sa v súčasnosti používa ako biomarker pri KVO, skúmali sme účinky GDF15 na cievnu a endotelovú funkciu. V *kapitole 5* sme ukázali, že GDF15 zohráva dôležitú úlohu v inhibícii cievnej kontrakcie a relaxácie v oboch skupinách divokých aj GDF15 knockout myší. Fenylefrínom vyvolané kontrakcie a acetylcholínom (ACh) sprostredkované relaxácie boli podobné u divokých a GDF15 knockout myší.

Predinkubácia s GDF15 inhibovala kontrakcie u oboch typov myší tým, že zvyšovala výdaj bazálneho oxidu dusného (NO). Taktiež inhibovala acetylcholínom vyvolanú relaxáciu u oboch skupín myší, a to znížením stimulácie uvoľňovania NO. Narušenie kaveolárnym blokátorom viedlo k podobnej inhibícii cievnych reakcií ako predinkubácia s GDF15 a kombinácia GDF15 s týmto blokátorom neviedla k ďalšiemu zníženiu cievnych reakcií. Zdá sa teda, že GDF15 poškodzuje endoteliálnu funkciu moduláciou endoteliálnej NO-syntázy prostredníctvom zmeny kaveolárnej signalizácie v endoteli.

V nasledujúcej kapitole (*kapitola 6*) sme sa zamerali na vzťah medzi GDF15 a endotelovým poškodením v obličkách, ktorý vedie k progresii albuminúrie u ľudí. Pre túto štúdiu sme použili starostlivo vybrané skupiny pacientov s príznakmi poškodenia a kontrolnej skupiny, pričom poškodenia boli definované zmenami v albuminúrii- prechodom vedúcim od normo- k mikro- a od mikro- k makro-albuminúrii. Pacienti v kontrolnej skupine s nezmenenou albuminúriou boli presne spárovaní podľa veku, pohlavia, hodnoty albuminúrie a priebehu trvania diabetu. Pozorovali sme dve rôzne populácie: skupinu subjektov s T2DM a skupinu subjektov s hypertenziou. Plazmatické hladiny GDF15 boli stanovené u oboch skupín pacientov na začiatku, tj pred prechodom do rozsahu zvýšenej albuminúrie. Základné hladiny GDF15 pred prechodom do albuminúrie boli vyššie v prípadoch poškodenia, než u kontrol. Okrem toho bola hladina GDF15 nezávisle spojená s progresiou albuminúrie u skupiny pacientov s T2DM a zlepšila predikciu progresie albuminúrie s vyššou hodnotou c-štatistiky a zlepšenou hodnotou integrovanej diskriminácie (IDI). Meranie hladiny GDF15 v sére bolo rozšírené na skupinu subjektov s hypertenziou bez diabetu. Naše výsledky ukazujú, že hladina GDF15 v sére môže predstavovať cenný prognostický ukazovateľ pri progresii albuminúrie a renálnej endotelovej dysfunkcie u pacientov s ochorením T2DM.

Naše údaje uvedené v danej práci poukazujú na dôležitú úlohu GDF15 v rozvoji diabetickej nefropatie. Lokalizácia GDF15 u diabetických potkanoch a myšiach bola identifikovaná v glomeruloch. Ďalšie štúdie s GDF15 knockout myšami a divokými myšami preukázali, že GDF15 zachováva tubulárnu funkciu a zabraňuje intersticiálnemu poškodeniu počas diabetu typu 1 i 2. Avšak GDF15 nechráni glomeruly pred sklerotizovaním a ani nezlepšuje hladinu albumínu v moči. Predinkubácia aorty s GDF15 vplýva na endotelovú funkciu, pretože zhoršuje cievnu kontrakciu a relaxáciu. Plazmatické hladiny GDF15 korelujú s progresiou albuminúrie u pacientov s T2DM. Poznatok, že BMPR2 je hlavný receptor

GDF15 aktivácie, môže identifikovať smer pri liečbe u T2DM a uľahčiť vývoj nových terapeutických postupov na obmedzenie poškodenia obličiek u diabetikov.

Záverečná diskusia

V našich štúdiách sme zistili, že GDF15 chráni tubuly obličiek a interstícium pri vývoji diabetického poškodenia. V predchádzajúcich experimentoch na srdci a na kultivovaných kardyomyocytov po vyvolaní infarktu myokardu a ischémie/reperfúzie boli preukázané ochranné účinky GDF15 a boli taktiež potvrdené v niekoľkých ďalších štúdiách na rôznych orgánoch a tkanivách po pôsobení škodlivých podnetov, vrátane chirurgických, toxických, ischemických a hypertoxických poškodení pľúc a obličiek, po chirurgických a chemických zraneniach či po tepelnom šoku pečene a ischémii žlčovodu.

Mechanizmy, ktorými GDF15 chráni tieto tkanivá a orgány sú rôznorodé. Inhibícia apoptózy buniek plyvom GDF15 bola zistená v endoteli, srdci, pľúcach a obličkách. Okrem toho GDF15 ovplyvňuje aj bunkovú proliferáciu v pľúcach a obličkách, čo naznačuje, že GDF15 môže byť zapojené do regenerácie špecifických typov buniek po poškodení. Pri reakcii na metabolickú acidózu GDF15 sprostredkováva regeneráciu epiteliálnych buniek v zberných kanálikoch v obličkách myší. GDF15 moduluje aktiváciu leukocytov v srdcovom endoteli, čím bráni zápalu. V našich experimentoch v modeloch diabetickej nefropatie sme potvrdili účinky GDF15 na proliferáciu a zápal, ale nenašli sme vplyv GDF15 na apoptózu.

Napriek ochranným vlastnostiam GDF15 v rôznych orgánoch a tkanivách, niekoľko vedeckých skupín objavilo koreláciu medzi hladinou GDF15 v plazme a morbiditou i mortalitou pri KVO aj diabete. Navyše naša štúdia ukázala, že zvýšené hladiny GDF15 v sére dokážu predpovedať progresiu albuminúrie u pacientov s T2DM (*kapitola 6*).

Zjavné protichodné účinky GDF15 v sére a v obličkách možno vysvetliť zisteniami opísanými v tejto práci. Po prvé, v *kapitole 2* a *3* sme preukázali, že zdrojom GDF15 bezprostredne po indukcii diabetu u potkanov a myší je práve glomerulus. V *kapitole 6* zase ukazujeme, že plazmatické hladiny GDF15 predpovedali priebeh glomerulárneho poškodenia u pacientov s T2DM. Zvýšené plazmatické hladiny GDF15 preto podľa všetkého odrážajú počiatočné glomerulárne poškodenie pri diabete. V *kapitole 4* dokazujeme, že interstícium a tubuly sú hlavné ciele pre ochranné pôsobenie GDF15 v diabetickej obličke. Genetické systémové odstránenie GDF15 neovplyvnilo diabetické

glomerulárne poškodenie, preto naše údaje naznačujú, že zdroje a ciele GDF15 sú odlišné. Plazmatické hladiny GDF15 môžu odrážať rozsah diabetického glomerulárneho poškodenia (čo nie je ovplyvnené GDF15), zatiaľ čo GDF15 poskytuje ochranu tkanivám, ako sú tubuly obličiek a interstícium.
Po druhé, v *kapitole 5* sme dokázali, že predinkubácia s GDF15 mala účinok na cievny endotel aorty, následkom čoho bola cievna kontrakcia a relaxácia inhibovaná, čo viedlo k zmenám vo funkcii cievneho endotelu aorty. Zhoršená relaxácia sprostredkovaná akútnym vplyvom GDF15 je podobná endotelovej dysfunkcii pozorovanej v niekoľkých modeloch KVO. Výsledky v našich pokusoch naznačujú, že zatiaľ čo GDF15 poskytuje ochranu poškodeným orgánom a tkanivám, dlhodobé zvýšenie hladiny GDF15 môže viesť k endotelovej dysfunkcii prispievajúcej k rozvoju KVO a ochoreniu obličiek.

Na záver môžeme zhodnotiť, že údaje uvedené v tejto práci poukazujú prvý krát na úlohu GDF15 v rozvoji diabetickej nefropatie. GDF15 chráni u diabetikov tubuly a interstícium obličiek a je prognostickým ukazovateľom progresie albuminúrie.

V nasledujúcich experimentoch sa môžeme zamerať na vývoj GDF15 knockout myší špecificky iba v obličkách a na presné objasnenie úlohy GDF15 v obličkách pri diabete. Úloha BMPR2 ako receptora pre aktiváciu GDF15 by mala byť skúmaná v iných tkanivách obličiek, vrátane tubulov. Identifikácia GDF15 receptora poskytuje totiž nový nástroj na ovplyvnenie jeho signalizačného systému, ktorý umožňuje vývoj moderných postupov v terapii diabetickej nefropatie.

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Magdaléna Mazagová was born on May 17th 1984 in Piešťany, Slovakia. In 2002, she completed secondary school at the Einsteinova gymnasium in Bratislava, Slovakia. In September 2002, she started her pharmaceutical studies at the Faculty of Pharmacy, Comenius University, Bratislava, Slovak republic. During the master study, she attended exchange student program, where she observed practicing skills in dispensing and work in pharmacy in Ottawa, Canada in july 2005, september 2006 and in pharmacy in Townsville, Australia in august 2006. In july 2007, she succesfully obtained Master of Science in Pharmacy degree. In november 2007, she started the work presented in this thesis at the department of Experimental Pharmacology, University Medical Center Groningen (Groningen, Netherland), under the supervision of Leo E. Deelman, PhD. After obtaining her PhD degree, she is focusing to continue in the field of preclinical and clinical research.