Myogenic constriction in renal failure – cause and therapy

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The cover photo represents vascular system with regulatory mechanisms that control blood pressure and thereby hemostasis of the body as the whole.

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Table of contents

Chapter 1 Introduction and the aims of the thesis ……..**9**

Chapter 2 Vascular smooth muscle function of renal glomerular and interlobar arteries predict renal damage in rat……..**21**

Chapter 3 Development of doxorubicin-induced proteinuria is predicted by healthy *in vivo* preglomerular arteriolar function……..…**41**

Chapter 4 Renal myogenic constriction protects the kidney from age-related hypertensive renal damage in the Fawn-Hooded rat…….…….**59**

Chapter 5 Losartan protects mesenteric arteries from ROS associated decrease of myogenic constriction after 5/6 nephrectomy……....**79**

Chapter 6 Vildagliptine restores renal myogenic function and attenuates renal sclerosis independently of effects on blood glucose or proteinuria in Zucker Diabetic Fatty rat…….…….…**101**

Chapter 7 Epidermal growth factor receptor inhibitor PKI-166 governs cardiovascular protection without beneficial effects on the kidney in hypertensive 5/6 nephrectomized rats……..**123**

Chapter 8 Summary and future perspectives…….…**147**

Samenvatting Summary in Dutch……**159**

Zhrnutie Summary in Slovak……...**165**

Acknowledgements, dankword, pod'akovanie …….…….....**171**

Chapter 1

Introduction

Myogenic constriction or the Bayliss effect: a 100 year old discovery

The myogenic constriction is defined as the ability of small arteries, called arterioles (and some veins, lymphatic vessels) to constrict with elevated intraluminal pressure and dilate, when pressure falls. The first report of this phenomenon is attributed to William Bayliss[1].

Sir **William Maddock Bayliss** (2 May 1860 – 27 August 1924) was an English physiologist. He was born in Wolverhampton, Staffordshire and gained a B.Sc. from London University. He graduated MA and DSc in physiology from Wadham College, Oxford. Bayliss and Ernest Henry Starling discovered the peptide hormone secretin - the first example of hormonal action of the intestines. He was involved in research in major areas of physiology, biochemistry, and physical chemistry[2]. Bayliss World War I investigation of wound shock led him to recommend gum-saline injections, a treatment that saved many lives[3]. In 1902 Sir William Bayliss made the observation that the transient increase of blood volume in organs (also in the kidney) occurred after brief periods of interruption of blood flow. He published these observations in the paper "On the local reactions of the arterial wall to changes of internal pressure"[4], where he for the first time described myogenic behavior in anesthetized animals and isolated arteries. This finding disclosed a novel paradigm in physiology, since its mechanism is independent of neural or humoral stimulation, therefore being of local origin. Later on, this mechanism was identified as an important regulator of blood flow in tissues. However, other researchers pointed towards the fact that vasodilatation could occur as a result of accumulation of vasodilator metabolites during the periods of interruption of blood flow. Nevertheless, in 1923, Bayliss published a monography, in which he stated: "On the whole, I fear we must regard the question as undecided."[5].

In 1949, the concept of myogenic constriction was revitalized by work of Bjorn Folkow[6] with pressure-flow experiments on whole organs and his work brought a satisfactory explanation of the pressure induced vascular response. Later on, grace to the development of techniques for microvascular preparation, the concept of myogenic constriction expanded until in the early 1990`s the discovery of stretch-sensitive ion channels[7] provided a new outlook on this phenomenon.

Mechanism of myogenic constriction

The processes involved in generation of myogenic constriction (MC) are summarized in Figure 1. The cascade involves the stimulus, *i.e.*, the increase in intraluminal pressure (stretch of vascular smooth muscle), followed by the detection systems that are responsible for mechanocoupling of the pressure/stretch signal through the membrane and translating this to intracellular events, leading to a signal transduction mechanism of kinases that finally cause phosphorylation of myosin light chain and contraction of vascular smooth muscle[1;8]. Intraluminal pressure (stretch of vascular smooth muscle) is the stimulus to trigger MC. A major deficiency in our understanding of myogenic signaling relates to how the initial stimulus is detected and how these events lead to membrane depolarization. Nevertheless, several mechanisms have been proposed so far: extracellular matrix–integrins interactions, cytoskeleton (actin filaments, intermediate filaments, microtubules, and C-fibers), mechano-sensitive ion channels and enzymes, specialized membrane domains (*e.g.* caveolae, lipid rafts). Recently, attention is heavily focused on the TRP channels – transient receptor potential channels *e.g.* – TRPC6, TRPC7, TRPM4, TRPV4, TRPV1[9;10], which seem to bring possibilities in the area of cellular triggers of the MC pathway, the part that is nowadays still well less explored. If proven true, this will expand possibilities to manipulate the initial steps of MC generation. Unfortunately, selective inhibitors or activators of these TRP channels are not yet available, and therefore research in this field is hampered. Activation of MC detection systems leads to depolarization of the cell and subsequent opening of voltage gated channels, causing calcium influx and activation of various second messenger pathways *e.g.* phospholipase C, inositol trisphosphate, diacylglycerol, protein kinase C, MAP kinase and 20-hydroxyeicosatetraenoic acid[11]. Moreover, calcium sensitization is importantly involved in the generation of MC[12]. Rho kinase dependent calcium sensitization involves the inactivation of myosin light chain phophatase (which dephosphorylates myosin light chain – making it inactive). Nevertheless, it seems that signaling pathways involved in MC are equally active over the whole pressure range and selective, or only partial activation, occurs in certain pressure ranges[13]. Moreover, it seems that MC pathways are vessel type specific, which increases the difficulty to establish the exact nature of these phenomena.

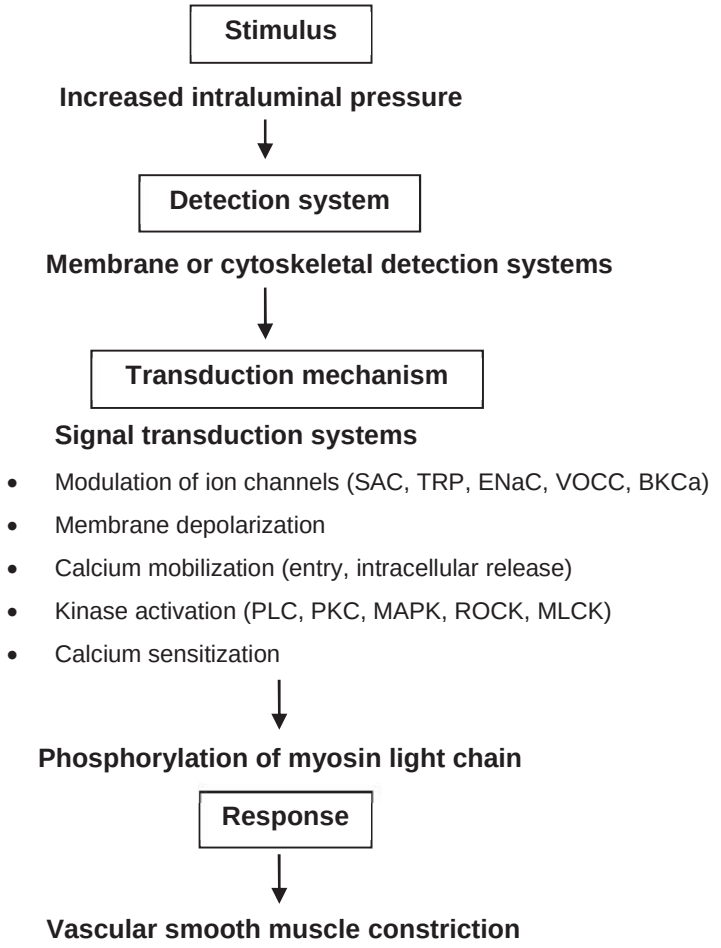

Figure 1. Schematic pathway of myogenic constriction generation of smooth muscle cell. Pressure as a stimulus to trigger myogenic constriction is detected by cytoskeletal detection systems and than further translated via transduction signaling involving: SAC – stretch activated channels, TRP – transient receptor channels, ENaC – epithelial Na+ channels, VOCC – voltage operated channels, BKCa – Large conductance, Ca2+-activated K+ channels, leading to membrane depolarization. Opening of voltage gated channels causing calcium influx and subsequent activation of kinase second messenger pathways: PLC - phospholipase C, PKC - protein kinase C, MAPK – mitogen activated protein kinase, ROCK – Rho – kinase, MLCK – myosin light chain kinase. (adapted from Hill, M.A. *et al*[11])

Renal myogenic constriction – part of the renal autoregulatory mechanism

The MC of renal vasculature was recognized as cornerstone of the autoregulation of blood flow in the kidney, in addition to tubuloglomerular feedback (TGF, which involves flow dependent signals sensed by macula densa mediating constriction/relaxation of the glomerular afferent and efferent arteriole thus adjusting pressure in the glomerulus). The phenomenon of renal autoregulation is the control of the way in which the kidney responds to changes in arterial pressure. The kidney is one of the organs which maintain a constant blood flow and glomerular filtration rate over a wide range of blood pressures. When systemic blood pressure is increased, leading to an increase in renal perfusion pressure, MC occurs in the preglomerular vasculature to protect the glomerulus from a barotrauma due to increased intraglomerular pressure. Therefore, MC serves as a safeguard to prevent transmission of pressure and its fluctuations into the glomerulus. If this system fails, the resulting increase in intraglomerular pressure ultimately leads to glomerular hypertension and subsequent renal damage.

Renal damage and hypertension

Increase in glomerular pressure is a central determinant of renal damage development[14]. Increased glomerular pressure, when persistent, leads to glomerular damage as manifested by glomerulosclerosis, proteinuria, particularly albuminuria[15] and - when untreated - ultimately to chronic renal failure (CRF). Nevertheless, the question is still unresolved why some patients with hypertension develop renal damage whereas others do not, genetic factors seem to play an important role, as illustrated by the five times greater chance to develop chronic renal failure in hypertensive African-Americans compared to hypertensives of Caucasian decent[16]. Possibly, this difference reflects a relatively impaired renal autoregulation/impaired MC in African-Americans, which allows hypertension to reach the glomeruli. On the other hand, renal disease itself can lead to hypertension[17], which in turn may affect autoregulation - thus creating a vicious circle.

Rat models of renal damage and hypertension

The 5/6th Nephrectomy (5/6Nx) model – model of surgically reduced nephron number

By ablation of 5/6th part of kidney tissue in the rat, progressive renal damage develops resulting in proteinuria, glomerulosclerosis and renin-associated hypertension. The 5/6Nx model is a model of progressive renal damage, where the initial reduction of nephrons ultimately leads to damage to the remaining ones[18]. The plasma renin levels in 5/6Nx rats suggest that the secretory rates for renin may be increased for remnant nephrons (perhaps to

keep constant glomerular filtration rate). Furthermore, with nephron reduction, renin clearance rate falls down[19] leading to elevated renin and consequently elevated Angiotensin II levels, which cause hypertension. Moreover, further elevations of blood pressure in this model (by high salt intake) exacerbate the development of renal damage[20]. Therefore, MC of preglomerular arteries plays an important role in preventing the transfer of elevated pressure into glomerulus.

The Fawn Hooded rat – model of myogenic constriction failure

An increase in blood pressure is common with increasing age[21], which also increases the risk of developing renal damage. Genetic and environmental factors play an important role in this process. The Fawn Hooded Hypertensive rat (FHH) is an example of impaired renal autoregulation in which systemic hypertension is transmitted into the glomerulus due to impaired MC of the renal preglomerular vessels while tubuloglomerular feedback is still intact[22]. This suggests that solely a dysfunction in MC accounts for renal hypertensive damage in the FHH rat. Conversely, the Spontaneously Hypertensive Rat (SHR) has substantial hypertension (systolic blood pressure around 200 mmHg), whereas renal damage is virtually absent. Ito *et al* indeed found an increased in renal myogenic response in SHR[23] when compared to a control strain, which may explain why the SHR does not develop renal damage. Thus, observations in both FHH and SHR emphasize the importance of patent MC in the protection of the kidney from hypertensive damage.

The Zucker Diabetic Fatty (ZDF) rat – model of type II diabetes mellitus

Type 2 diabetes mellitus (T2DM) is associated with an increased risk of both micro- and macrovascular complications[24;25], along with cardiovascular disease leading to end organ damage, such as chronic renal failure (CRF)[26;27], manifested as proteinuria and glomerulosclerosis. The Zucker Diabetic Fatty[28] (ZDF) rat is a substrain of the obese Zucker rat, which develops diet-induced type-2 diabetes (T2DM) and metabolic syndrome, however without hypertension. These rats were selected for studies described in this thesis because they display similar conditions as T2DM in humans: obesity , hyperglycemia and an abnormal lipid profile[29], thereby representing a clinically relevant model. In those rats impaired renal autoregulation was observed as well[30].

Adriamycin nephrosis

Adriamycin (doxorubicin) is a cytostatic drug to which certain types of cancer respond. Of particular interest is that a single injection of adriamycine causes CKD in rat, manifested by severe proteinuria and glomerulosclerosis[31].

MC on the periphery – mesenteric artery

Mesenteric artery has become very frequent research tool to investigate the peripheral vasculature. In the mesenteric vascular bed no overt autoregulation occurs, but MC of mesenteric arteries represents regulation of peripheral resistance. Moreover, from the perspective of localized or generalized myogenic dysfunction, investigation of MC of mesenteric arteries is very useful.

Renal interlobar arteries

The isolated renal arteries studied in this thesis are interlobar arteries, which represent larger arteries upstream of the afferent and efferent glomerular arteriole. It is largely unknown to what extent these arteries contribute to renal autoregulation or preglomerular resistance in health and different disease models. Given the size and environment of interlobar arteries, it is assumable that changes at these levels reflect those at the level of glomerular arterioles.

Predictive value of vascular function in susceptibility to renal failure

The concept that the functions of isolated renal arteries predict susceptibility of an individual to renal damage is not new. As shown before in our lab, endothelium-mediated relaxation of isolated interlobar arteries at a healthy stage (isolated from the kidney obtained at 5/6Nx) predict the proteinuria and glomerulosclerosis that develops thereafter[32]. However, various endothelial derived components not only influence vasomotor properties, but also affect inflammatory responses, including NO and prostaglandins [33]. Both factors, *i.e.*, blood flow control and anti-inflammatory properties may be involved in this predictive property of endothelial function. Nevertheless, the above mentioned predictive observation suggests that pre-existing vascular integrity is one of the key factors in susceptibility to develop CKD. Examination of vascular smooth muscle properties in this respect may disclose the contribution of blood flow regulation.

Aim of the thesis

Therefore, the aim of this thesis was to investigate the role of arterial vascular smooth muscle function, particularly of MC, in chronic kidney disease rat models and was therefore named "Myogenic constriction as cause and therapy of CKD. The main topics that were addressed:

1. Study MC to investigate whether
    a. higher MC of preglomerular vessels is associated with lower vulnerability of kidneys to damage in several rat models: reduced renal mass/hypertension – 5/6Nx (**chapter 2**), type 2 diabetes mellitus – ZDF rat (**chapter 6**), and in ageing induced hypertension with predisposition to kidney damage (**chapter 4**)
    b. MC of mesenteric arteries is affected by kidney disease with and without hypertension (**chapters 5, 6, 7)**
    c. pharmacological treatment of kidney disease with and without hypertension influences MC (**chapters 5, 6, 7**)
2. to identify whether *in vivo* glomerular vascular contractility to Angiotensin II predicts individual susceptibility to CKD in the adriamycine model and in 5/6Nx model and moreover in 5/6Nx model how it is related to MC (**chapter 2, 3**)

In **Chapter 2** we examined whether animals with a better MC of renal interlobar arteries at baseline (obtained at 5/6Nx) are protected against subsequent hypertensive renal damage compared to animals with lower baseline MC. Moreover, using intravital microscopy we investigated whether this concept of healthy vascular function relating to renal damage holds true *in vivo* at the level of preglomerular afferent- and efferent vessels in 5/6Nx. In **Chapter 3**, we examined the relationship between contractile responses of preglomerular arterioles to angiotensin II before adriamycin induced proteinuria thereafter. In a previous study[34], we identified the Fawn-Hooded rat as an interesting model. "The FHH has loss of myogenic tone, high blood pressure and renal damage, whereas FHL has none of these". However, as aged FHL develops hypertension to a similar level as FHH, we investigated MC of renal arteries to substantiate its role in development of renal damage (**Chapter 4**). In the 5/6Nx model, intervention in the RAAS is effective in slowing down the progression of renal damage, indicating the pathogenic role of angiotensin II in this model[35;36]. Detrimental effects of angiotensin II are generally conveyed through the angiotensin receptor type 1 mediated activation of second messenger systems. Partly, this seems dependent on angiotensin type 1 receptor transactivating the epidermal growth factor receptor[37]. Therefore, in **Chapter 5** and **Chapter 7,** we examined the effect of treatment of

5/6Nx rats in relation to MC. Both the angiotensin receptor type 1 blocker losartan (**Chapter 5**) and PKI-166, an epidermal growth factor receptor blocker (**Chapter 7**), were examined. We aimed to explore whether these drugs were able to slow down the renal damage progression and how it would affect the reduction in MC of peripheral (mesenteric) arteries observed in 5/6Nx [36]. Finally, in **Chapter 6,** we studied changes in MC of renal and peripheral arteries in a diabetic model, the ZDF rat and treatment with the DPP-IV inhibitor vildagliptine, which has been described to encompass both blood glucose lowering and additional protective properties on the cardiovascular system.

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Chapter 2

Vascular smooth muscle function of renal glomerular and interlobar arteries predict renal damage in rat

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Abstract

Background: Susceptibility to renal injury varies among individuals. Previously, it was shown that individuals with good baseline (a priori) endothelial dilatory ability in isolated (*in vitro*) renal arterioles developed less renal damage after 5/6 nephrectomy 5/6Nx. In this study, we investigated whether pre-existing renal vascular integrity predicts subsequent renal damage after 5/6Nx, using *in vivo* intravital microscopy and *in vitro* myogenic constriction of small renal arteries.

Methods: Anaesthetized rats underwent intravital microscopy to visualize constriction to angiotensin II (Ang II) (30 ng/kg/min) of glomerular afferent and efferent arterioles, with continuous measurement of blood pressure, heart rate and renal blood flow. Thereafter, 5/6Nx was performed and interlobar arteries (ø 261±2 µm) were isolated from the extirpated kidney and myogenic constriction was assessed in a perfused vessel setup. As follow up, blood pressure and proteinuria were assessed weekly for 12 consecutive weeks and focal glomerulosclerosis (FGS) was determined at the end of study.

Results: Infusion of Ang II induced significant constriction of both afferent and efferent glomerular arterioles ($p<0.001$ compared to baseline – saline infusion), which correlated strongly with proteinuria ($r = 0.73$; $p = 0.01$ and $r = 0.90$; $p = 0.01$, respectively), and FGS ($r = 0.691$; $p = 0.019$; $r = 0.664$; $p = 0.026$, respectively) at 12 weeks after 5/6 Nx.
Furthermore, *in vitro* measured myogenic constriction of small renal arteries inversely correlated with proteinuria ($r = - 0.71$, $p = 0.02$) and FGS ($r = - 0.882$; $p = 0.01$) 12 weeks after 5/6Nx. Moreover *in vivo* vascular reactivity inversely correlated with *in vitro* reactivity (afferent: $r = - 0.682$ $p = 0.021$; efferent: $r = - 0.618$ $p = 0.043$)

Conclusion: Both *in vivo* afferent and efferent responses to Ang II and *in vitro* myogenic constriction of small renal arteries in the healthy rat predict the severity of renal damage induced by 5/6Nx. Intraorgan vascular integrity may provide a useful tool to guide prevention and treatment of renal end-organ damage.

Introduction

While the susceptibility to develop renal damage varies considerably among individuals, its determinants are still incompletely understood. The extent of existing systemic factors, such as diabetes or hypertension, cannot fully explain the predisposition to renal failure[1], suggesting additional factors to be involved. In general, the factors governing this individual vulnerability to renal damage are thought to be intrinsic to the kidney and probably largely genetically determined[2].

We have previously shown that individual differences in the endothelial dilative capacity of isolated small renal arteries of the healthy animal strongly predicts the extent of renal damage in various models of experimental renal disease, *i.e.*, 5/6 nephrectomy (5/6Nx)[3], unilateral nephrectomy combined with myocardial infarction[4] and adriamycin nephrosis[5]. These observations suggest that the patency of renal endothelial function is a factor determining the severity of damage after induction of the disease.

Control of intraglomerular pressure is essential to maintain kidney health. Intraglomerular pressure is controlled mainly by the Renin Angiotensin Aldosterone System (RAAS), tubuloglomerular feedback and myogenic constriction (MC), all acting on the regulation of the vascular tone of the afferent and efferent arteriole of the glomerulus. On the other hand, the RAAS plays a key role in the progression of renal damage such as after 5/6Nx, through elevation of systemic blood pressure thereby increasing intraglomerular pressure by unbalanced constriction of the efferent and afferent arteriole. Whether contractility of the afferent or efferent arteriole to Ang II determines development of renal damage in this model remains unclear.

Angiotensin II in renal damage in the 5/6Nx model

The 5/6Nx model of reduced nephron number is a model of progressive renal damage, where the initial reduction of nephrons ultimately leads to damage to the remaining ones[6]. The plasma renin levels in 5/6Nx rats suggests that the secretory rates for renin may be increased for remnant nephrons and furthermore with nephron reduction, renin clearance rate falls down[7]. Taken together, these phenomena lead to elevated renin and consequently Ang II levels as well as subsequent development of renal damage after 5/6 Nx. Intervention in the RAAS is effective in slowing down the progression of renal damage in this model which collectively point on the pathogenic role of Ang II in this model[8;9]. The importance of RAAS is further substantiated by experiments showing that sustained Ang II administration dose-dependently induces proteinuria accompanied by glomerular damage

otherwise healthy individuals[10], while short-term Ang II infusion, sufficient to affect renal hemodynamics, does not elicit proteinuria[11].

Previous studies showing the relationship between healthy interlobar artery function and renal damage employed *ex vivo* isolated pre-glomerular renal arteries. To investigate whether functional aspects of glomerular afferent and efferent arteriole can determine/predict the damage thereafter, we studied their responsiveness to Angiotensin II in *in vivo* experimental conditions[12;13] prior to the induction of renal damage upon 5/6Nx. Furthermore, we sought to test whether myogenic constriction, assessed *in vitro* in vessels obtained at 5/6Nx, predict the damage thereafter. Moreover, we aimed to find a relationship between these two entities, i.e. *in vivo* glomerular vascular contractility and *ex vivo* interlobar artery myogenic contractility to pressure.

Materials and methods

Experimental animals

Male Wistar rats (Hsd.Cpb.Wu, n = 11; 200-250g; Harlan, Zeist, the Netherlands) were used and housed under standard conditions (day/night rhythm 12h:12h, group housing in macrolon cages) approved by the institutional animal ethical committee. The rats had free access to food and drinking water and received a normal diet (RMH-B, 2181; ABDiets, Woerden, the Netherlands). After arrival in our laboratory, the rats had an acclimatization period for one week to get used to their new environment. After this period, the rats were trained for systolic blood pressure measurements (*i.e.* accustomed to immobility in a warmed restrainer for at least ten minutes), because stress would cause elevation of blood pressure. All animal experiments were conducted in accord with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

In vivo study

Intravital microscopy

The experimental setup (Fig. 1) consisted of a pencil video microscope with a corn shaped lens (optical magnification 3.5x) and a CCD camera (Nihon Kohden, Tokyo, Japan), a micromanipulator, a xenon light source (LB-18; Welch Allyn, Tokyo, Japan), a monitor

(KLV-17HR1; Sony, Badhoevedorp, the Netherlands), a DVD recorder (RDR-GX7), and a computer for image analysis (Intel Pa). The lens was fitted with a 12.7 mm greyscale CCD image sensor (XC ES55L; Toshiba, Tokyo, Japan) at the focal length (200 mm) of the lens). A green filter to complement red was placed in front of a CCD image sensor to enhance the contrast on the monitor between vessels and peripheral tissue. The CCD image sensor was connected to the camera module (DC700; Sony, Tokyo, Japan) and images were recorded as stacked image film (60 frames/min). The final spatial resolution of the video microscope was confirmed to be 0.86 mm with magnification of 520x. This technique thus allows for full visualization of one glomerulus and its relevant surrounding region in each experimental protocol [6; 7].

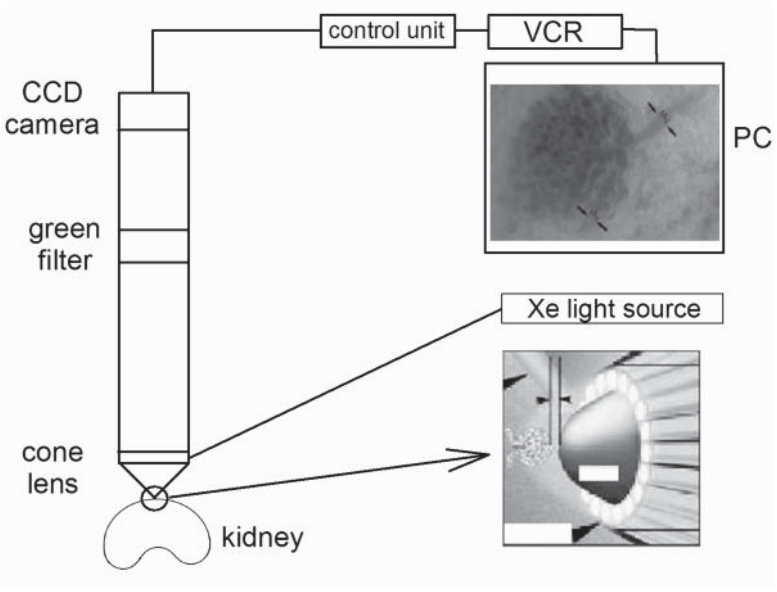

Figure 1. *Schematic picture of experimental of intravital microscopy consist of pencil video microscope with corn shaped lens, micromanipulator, xenon light source, monitor a DVD recorder and computer for image analysis.*

Analysis of vascular diameters

The image at each time point was captured as a stack of 60 frames using an image capture board (LG-3; Scion Computer Service, Frederick, Maryland, USA) installed on the image analysis computer. At least 3 clear frames, which were not influenced by respiration and heartbeat, were selected from the 60 frames in the captured stacks and analyzed. Diameters of afferent and efferent glomerular arteries were measured using image software (Scion Corporation, Frederick, Maryland, USA) after calibration of the number of pixels (scale 2.42 pixels per μm).

Experimental protocol

Before the experiment started, the rats were anesthetized with isoflurane/O_2 (2.5% isoflurane; Pharmachemie BV Haarlem, The Netherlands) to insert a catheter (BD Insyte-W 24 GA 0,75IN – 0,7x19 mm) in the tail vein. This catheter was used to administrate a bolus injection of 36 mg/kg pentobarbital sodium (Hospital Pharmacy UMCGroningen, the Netherlands) once isoflurane administrationwas ended and the animal started to show reflexes. The operation region was anaesthetized with lidocain (20 mg/ml; Fresenius Kabi, Germany).

The carotid artery was cannulated for measurements of systolic blood pressure, diastolic blood pressure, heart rate using a pressure transducer (Edwards Life sciences S.A., Saint-Prex, Switzerland) and an amplifier (model AP641G; Nihon Kohden, Tokyo, Japan). The jugular vein was cannulated for infusion of saline and angiotensin II. Then, the abdomen was opened by midline incision and the left renal artery was equipped with an ultrasonic flow probe (model 1RB; Transonic Systems, Ithaca, New York, USA) to measure renal blood flow (RBF), continuously registered with a flow meter (model T106; Transonic Systems). To expose glomeruli, the capsule of the renal cortex was removed, and a thin slice of the renal surface was removed (maximum depth 0.5 mm) using a scalpel. The tip of the pencil-probe charge coupled device (CCD) video microscope was guided to the bottom of the excision. Glomeruli in which the afferent and efferent arterioles could be visualized without affecting the blood flow were used. Per experiment, one glomerulus of the left kidney was monitored.
The protocol consisted of a baseline of 10 minutes saline infusion. Thereupon, angiotensin II infusion (10 min, 30 ng/kg/min; Bachem Bioscience) was started. Movies were recorded during steady state saline and angiotensin II infusion for later analysis. After this period, the

angiotensin II infusion was switched to saline for 30 minutes (in order to allow for return to baseline values) after which 5/6Nx was performed by removing the right kidney and by ligating two or three branches of the renal artery of the left kidney leading to infarction of approximately 2/3rd of this kidney. Animals received a subcutaneous injection of buprenorphine (10 µg/kg) postoperatively.

In vitro study

In vitro perfusion set-up

Small renal interlobar arteries with an intraluminal diameter of 261±2 µm were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously [14]. Artery segments were cannulated on glass micropipettes and the vessel chamber was continuously recirculated with warmed (37°C) and oxygenated (5% CO_2 in O_2) Krebs solution with a pH of 7.4. An inverted light microscope attached to a video camera and video dimension analyzer was used to continuously register lumen diameter.

Myogenic reactivity of small renal resistance arteries

Intraluminal pressure was set at 60 mmHg, arteries were allowed to equilibrate for 45 minutes and checked for smooth muscle and endothelium viability by a single dose of phenylephrine (PE, $3x10^{-7}$ mol/l) and acetylcholine (ACh; $3x10^{-5}$ mol/l), respectively. To exclude possible any influence of endothelium, arteries were mechanically denuded of endothelium. Removal of endothelium was confirmed by absence of dilative response to ACh ($3x10^{-5}$ mol/l) following a submaximal pre-constriction with PE ($3x10^{-7}$ mol/l).

Following a wash out, intraluminal pressure was decreased to 20 mmHg and myogenic reactivity was studied by obtaining active pressure-diameter curves over a pressure range of 20-160 mmHg in steps of 20 mmHg. Each pressure step was maintained for 5 minutes to reach the stable contractile response. Thereafter, calcium containing Krebs solution was exchanged for calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether)tetraacetic acid (EGTA, 2 mmol/l) and passive pressure-diameter curves were obtained over the same 20-160 mmHg pressure range.

Follow-up measurements of proteinuria and blood pressure

Urinary protein excretion was measured in samples of 24-hours urine collected when the rats were put in metabolic cages for 24h on a weekly basis for 12 weeks. Urinary protein concentration was determined by nephelometry (Dade Behring III, The Netherlands).

Weekly systolic blood pressure measurements were carried out in conscious animals with tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA).

Renal histology

Paraffin embedded kidneys were cut in 3 μm sections and stained with periodic acid Schiff (PAS) and the incidence of focal glomerulosclerosis (FGS) was microscopically evaluated according to standard procedures as described previously[15].

Statistical analysis

The results are expressed as mean ± SEM. Differences between the time periods were calculated and for comparisons a paired–sample t-test and independent t-test were used (SPSS, Inc.). Graphs were made using Sigma Plot and the relationship between parameters was calculated using regression analysis (SPSS, Inc.).

Area Under Curve (AUC) of myogenic tone was determined in individual arteries (Sigma Plot) and expressed in arbitrary units. Differences were considered significant at $p<0.05$.

Results

Systolic Blood Pressure, Proteinuria, Focal glomerulosclerosis

Systolic blood pressure, as measured in conscious animals, increased from 124 ± 3 mmHg (week 0) to 176 ± 12 mmHg 12 weeks after 5/6 Nx (Figure 2A). After 5/6Nx, proteinuria progressively increased within time from 37,5 ± 4,6 mg/24h at baseline to 229.1 ± 43.2 mg/24h 12 weeks after 5/6 Nx (Figure 2B). Renal damage after 12 weeks of 5/6Nx was evidenced further by a 42.8 ± 4.8 % incidence of focal glomerulosclerosis (FGS).

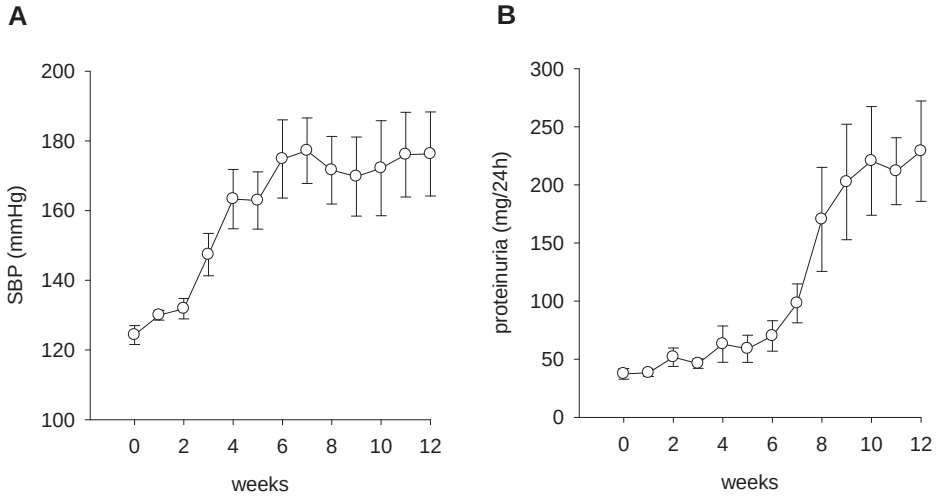

Figure 2. A) *The development of systolic blood pressure (mmHg) and* ***B)*** *proteinuria (mg/24h) in time (weeks) after 5/6 nephrectomy. Data are given as means ± SEM*

Acute changes of hemodynamic parameters and renal blood flow

To assess the Angiotensin II response, hemodynamic parameters were compared between baseline and at 10 minutes of Ang II infusion. Compared to baseline values, Ang II significantly ($p<0.05$) increased systolic blood pressure (23.9 ± 2.2%) and diastolic blood pressure (20.1 ± 2.9%), and reduced RBF by 24.2 ± 6.5%.

Intravital microscopic analysis of afferent and efferent glomerular arterioles

To evaluate changes in glomerular vascular reactivity, the changes in diameter of the afferent and efferent arterioles of the glomerulus were assessed (Figure 3A). Ten minutes of Ang II infusion caused a significant ($p<0,001$) constriction in both afferent and efferent arterioles when compared to saline infusion. Notably, no difference was observed in the of

afferent and efferent arterioles in response to Ang II infusion, as both were constricted to the same extend (Figure 3B).

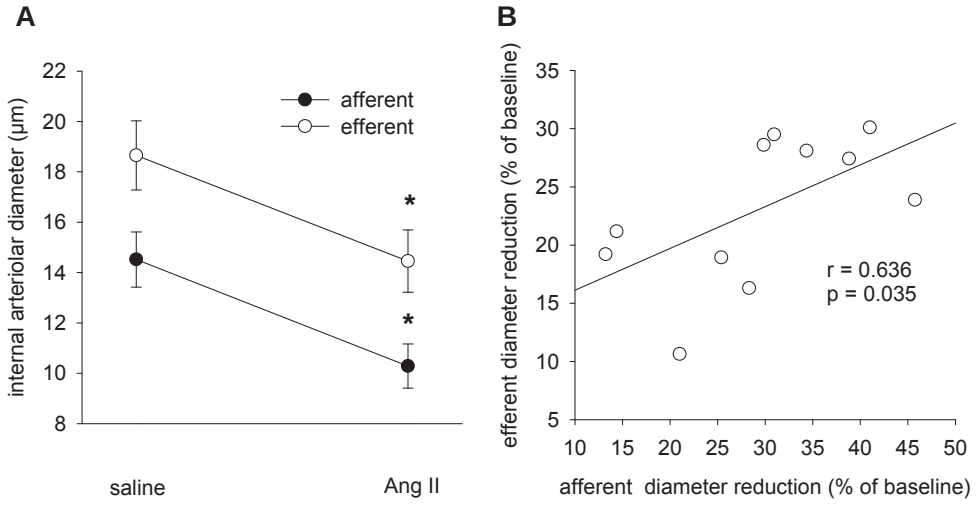

Figure 3. A) *Contractile response after Ang II (30 ng/kg/min) infusion of afferent and efferent arteriole (µm),* ***B)*** *correlation analysis between contractile response to Ang II of afferent and efferent arteriole. Data are presented as mean + SEM. * p<0,01 vs saline*

Myogenic constriction

Renal arteries isolated from the extirpated kidney at 5/6Nx from different rats developed myogenic constriction to a variable extend. Averaged myogenic constriction (MC) was 15.1±1.8 % of maximal MC over the whole pressure range (AUC: 1267 ± 114 arbitrary units) with individual values ranging from 5.8 % max MC (AUC: 586 arbitrary units) to 23.7 % max MC (AUC: 1927 arbitrary units).

Correlation analysis

The responsiveness of both afferent and efferent arteriole to Ang II measured before 5/6Nx *in vivo* predicted proteinuria and glomerulosclerosis (FGS) 12 weeks thereafter. The responsiveness of both afferent and efferent arterioles strongly correlated with proteinuria at week 12 after 5/6 Nx (afferent: r = 0.727; p = 0.011, Figure 4A; efferent: r = 0.900; p = 0.010, Figure 4B). Likewise, there was a strong correlation between Ang II responsiveness and FGS (afferent: Figure 4C r = 0.691; p = 0.019; efferent: Figure 4D, r = 0.664; p = 0.026). Therefore, these data indicate that healthy animals with higher responsiveness of glomerular arterioles to Ang II develop excess renal damage following subsequent 5/6 nephrectomy.

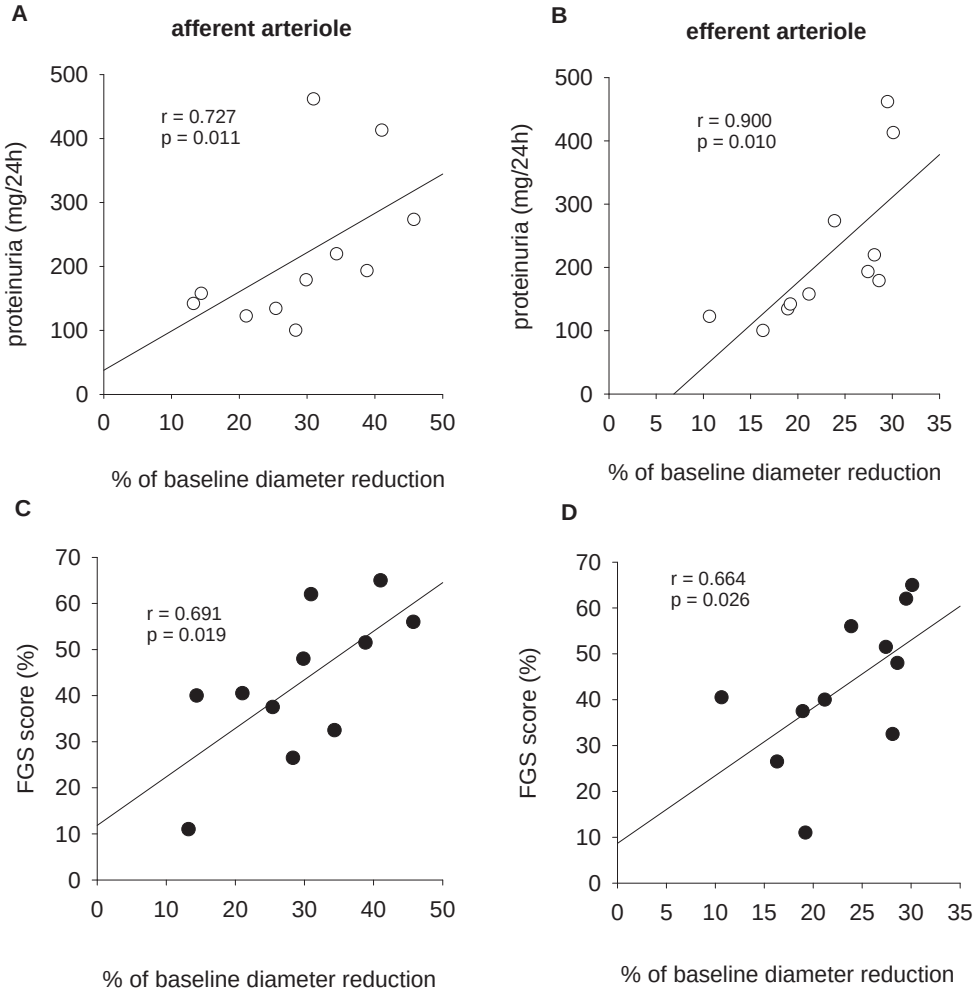

***Figure 4**. Correlation between individual Ang II (30ng/kg/min) induced responses at the time of 5/6Nx of the afferent and efferent arteriole and **A, B)** proteinuria (mg/24h), **C, D)** incidence of focal glomerulosclerosis (FGS) 12 weeks after 5/6Nx.*

Also, the myogenic constriction measured *in vitro* from renal small arteries obtained at 5/6 Nx predicted the proteinuria and FGS 12 weeks thereafter. Myogenic constriction negatively correlated both with proteinuria (Figure 5A, r = - 0.700; p = 0.016) and glomerulosclerosis (Figure 5B, r = - 0.882; p = 0.01), indicating that animals with a

pronounced baseline myogenic constriction assessed at 5/6 Nx developed lower proteinuria and FGS 12 weeks thereafter.

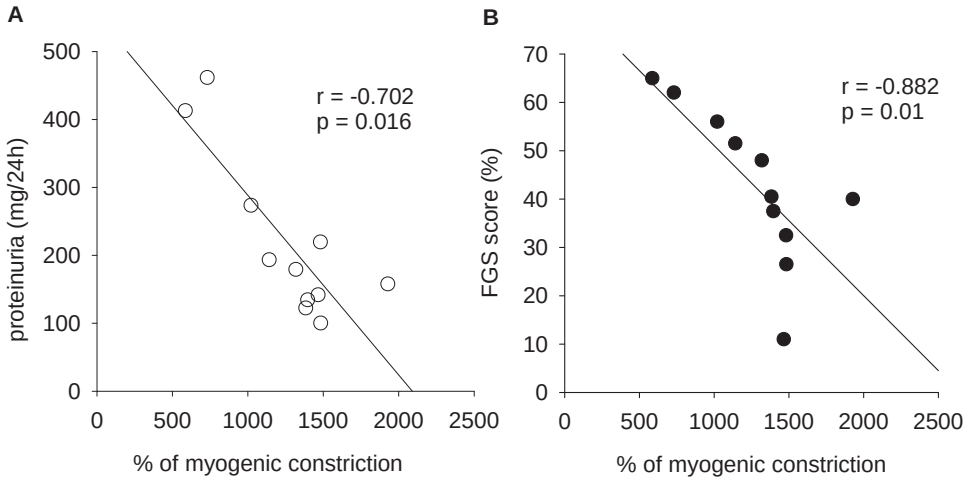

***Figure 5**. Correlation between individual myogenic constriction responses (expressed as AUC from individual curves) at the time of 5/6Nx and **A)** proteinuria (mg/24h), **B)** incidence of focal glomerulosclerosis 12 weeks after 5/6Nx.*

To substantiate a possible relationship between AngII sensitivity and the extent of MC, additional correlation analysis was performed. Importantly, a strong negative correlation was found between the *in vivo* reactivity of afferent and efferent arteriole and *ex vivo* assessed myogenic constriction (Figure 6, afferent: r = - 0.682 p = 0.021; efferent: r = - 0.618 p = 0.043). Thus, animals with lower responsiveness to Ang II displayed higher myogenic constriction.

To demonstrate a possible relationship between change of systolic blood pressure and contractile responses of afferent and efferent arteriole after Ang II infusion, correlation analysis was performed. No significant relation was found there (afferent: r = 0,218 p = 0,519; efferent: r = 0,291 p = 0.385).

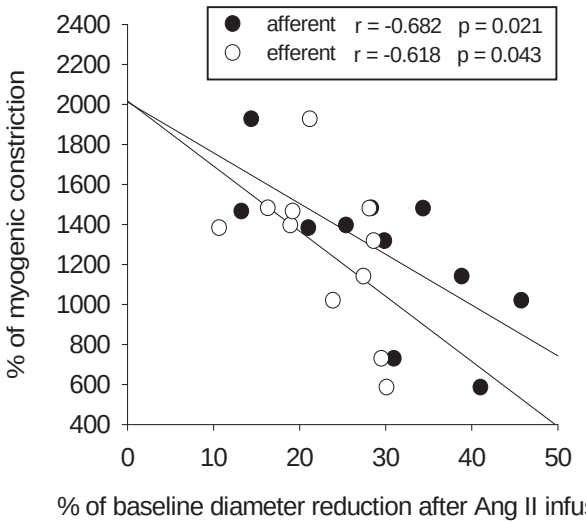

***Figure 6**. Correlation between individual myogenic constriction responses (expressed as AUC from individual curves) and reactivity to Ang II (30 ng/kg/min) of afferent and efferent arteriole at the time of 5/6Nx.*

To investigate whether contractile responses of afferent and efferent arterioles to Ang II predict the development of hypertension at 12 weeks after 5/6 Nx, correlation analysis was performed, which showed no significant correlation (afferent: r = - 0,077 p = 0,821; efferent: r = 0,005 p = 0,989). Moreover, myogenic constriction of isolated arteries also did not predict the development of hypertension at 12 weeks after 5/6 Nx (r = 0,068 p = 0,842).

Discussion

In the present study, we showed that *in vivo* reactivity of glomerular arterioles to systemically administered Ang II and *ex vivo* potential to develop myogenic constriction in small renal arteries of healthy rats predict the extent of renal damage 12 weeks after 5/6 Nx. These results extend our previous observations on the predictive property of *in vitro* assessed endothelial dependent dilation of healthy rat in small renal arteries[16]. Moreover, we show for the first time that healthy *in vivo* glomerular vascular reactivity to Ang II predicts the degree of renal damage after nephron reduction. Additionally *in vivo*, both afferent and efferent glomerular arteries react similarly to Ang II. Finally, an inverse relationship between *ex vivo* contraction of glomerular vessels to Ang II and *in vivo* myogenic contractility was found. Together, our data demonstrate that normal vascular

contractile function of renal arteries both on levels of glomerular arterioles and preglomerular arteries of healthy individuals predict renal hypertensive damage caused by 5/6Nx.

Additionally, the *in vivo* method we used in present study is clinically even more relevant compared to our previous studies done *ex vivo* and on larger preglomerular vessels. Assessment of intrarenal vascular function may offer possibilities as a diagnostic tool to determine susceptibility to progression of renal failure.

The role of afferent and efferent arteriole in glomerular pressure regulation

We observed increased renal damage in those animals that had reacted more to Ang II prior to 5/6Nx both on the level of afferent and efferent glomerular arteriole. The most obvious explanation is that the relationship reflects the sensitivity of individuals to Ang II, particularly as renal damage in the 5/6Nx model is (partly) driven RAAS activation. Glomerular pressure is a central determinant of renal damage development[17]. In transgenic rats harboring the mouse Ren2 renin gene, increased AT_1 receptor binding was found in vascular smooth muscle of afferent and efferent arterioles. This might suggest that up-regulation of AT_1 receptors contribute to the glomerular damage in these rats[18]. Therefore similarly to our situation, rats with higher AT_1 receptors expression that had reacted more to Ang II developed higher damage thereafter.

Myogenic constriction as a predictor of renal damage in 5/6 nephrectomy

We additionally show that rats with higher myogenic constriction assessed prior to 5/6 Nx developed less proteinuria and glomerulosclerosis than those with lower basal myogenic constriction. This observation is in keeping with the notion that myogenic constriction protects from an increase in intraglomerular pressure in rats with elevated blood pressure.

Increased intraglomerular pressure is a key determinant of renal damage development dictated by the integrity of the preglomerular vasculature[19]. This view is substantiated by observations in an animal models of renal failure (Fawn Hooded Hypertensive rat), in which a loss of myogenic constriction of interlobar arteries precedes renal damage which develops only when systemic blood pressure increases[20]. In contrast, Spontaneously Hypertensive Rats (SHR), who have high systemic blood pressure do not show renal damage. As SHR displays strong myogenic response of preglomerular vessels, this possibly is the reason why their kidneys are protected from hypertension[21].

Relationship between Ang II mediated constriction and myogenic constriction

Interestingly, we found a strong inverse relationship between myogenic contractility of *ex vivo* preglomerular vessels and *in vivo* Ang II mediated contractility of the afferent and efferent arterioles. There may be several explanations. The systemic action of Ang II infusion in this study caused an increase in systemic blood pressure, which most likely led to an increase in renal perfusion pressure as well, thus activating myogenic constriction. Moreover, non-pressor doses of Ang II strongly augment MC as measured in isolated mesenteric arteries[22]. Therefore, Ang II mediated constriction of the afferent and efferent arterioles may partly reflect the vessels ability to generate myogenic constriction. Our results, however, show the two parameters to be negatively correlated rather than positively, making this option unlikely. Secondly, Angiotensin type 1 receptor (AT1R) has been implicated as a stretch sensor. Mechanical stretch induces association of the AT1R with Janus kinase 2, translocation of G proteins into the cytosol, activation of ERK and production of inositolphophates[23]. These features imply that AT1R may act as a stretch receptor in vascular smooth muscle and hence be involved in MC generation. Indeed, we previously showed interaction of AT1R and MC in model of heart failure, where increased MC was restored by inverse agonists of the AT1R[24;25]. Moreover, we showed the increased MC to coincide with a decreased number of caveolae in vascular smooth muscle and that a similar increase in MC is induced by disruption of caveolae[26]. Thus it appears that the membrane distribution of the AT1R affects its responsivity to stretch. While being an attractive explanation, there is unfortunately too limited additional data to substantiate this hypothesis and further research is needed.

Similar responsiveness of afferent and efferent arteriole to AngII

We found no significant difference in the Ang II mediated constriction between the afferent and efferent arteriole. This observation is in line with the similar reactivity of afferent and efferent arteriole to Ang II found in a previous study employing intravital microscopy in a similar experimental settings[27]. Nevertheless, it is generally believed that the efferent arteriole displays stronger contraction to Ang II, resulting in an increased glomerular pressure to achieve physiological GFR. Reviewing the literature, a large discrepancy exists regarding extend of Ang II mediated constriction in afferent *vs.* efferent arteriole. There is substantial evidence that under physiological conditions Ang II predominantly constricts the efferent arteriole. Nevertheless, a similar amount of evidence identifies the afferent arteriole as the predominant glomerular vessel sensitive to Ang II[28;29]. Different experimental approaches or animal strain specificity may account for this discrepancy. However, in the current study, no significant differences of responses between the two

arterioles were detected although the trend (borderline significance) showed slightly higher contractility of the afferent arteriole to Ang II in our - *in vivo* - settings which supports the view that the afferent arteriole reacts more on Ang II rather than the efferent arteriole.

Conclusion

In the present study, we provide evidence that *in vivo* reactivity of afferent and efferent arteriole to systemic infusion of Ang II predicts the degree of renal damage induced by subsequent 5/6 Nx. Furthermore, *ex vivo* myogenic constriction of interlobar arteries isolated at induction of damage, i.e. 5/6 Nx, predicts subsequent renal damage as well. These observations imply that measurement of intrarenal vascular function may be used to identify individuals prone to renal impairment. The relationship between *in vivo* sensitivity to Ang II and future renal damage will facilitate the employment of renal vascular responsiveness in the clinical setting. Nevertheless, further research is needed to explain the molecular and/or genetic background of patent vascular function as determinant of susceptibility to renal damage.

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Chapter 3

Development of doxorubicin-induced proteinuria is predicted by healthy *in vivo* preglomerular arteriolar function

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Abstract

Background: Susceptibility to renal injury varies among individuals. Previously, it was shown that individuals with highly patent baseline endothelial dilatory ability in isolated renal arterioles developed more renal damage after doxorubicin injection. Whether *in vivo* pre-existing vascular integrity also predicts subsequent renal damage to doxorubicin is subject of this study. To this end, using *in vivo* intravital microscopy, baseline diameter and constrictive ability of afferent and efferent glomerular arterioles to angiotensin-II (ANGII) were measured and related to development of proteinuria following subsequent doxorubicin injection. Secondly, it was studied whether co-infusion with ANGII limits during doxorubicin injection limited long-term renal.

Methods: Anesthetized rats underwent intravital microscope to envision glomerular afferent and efferent arterioles. After stabilization with a saline infusion, ANGII (30 ng/kg) was infused in the experimental group or saline controls. Thereafter, renal damage was induced in both groups by doxorubicin injection in the tail vein continued on either saline or AngII. During the intravital protocol, arterial blood pressure, heart rate and renal blood flow were measured. Images of glomeruli were recorded for measurements of changes in vascular diameter of the afferent and efferent glomerular arterioles. After surgery, animals were followed for 6 weeks for measurement of blood pressure and proteinuria.

Results: ANGII infusion significantly changed all measured hemodynamic parameters except heart rate from baseline and similarly when compared to the control group. Infusion of ANGII also induced significant contraction of both afferent and efferent glomerular arterioles. Linear regression analysis between the change in afferent and efferent glomerular arteriolar diameter upon ANGII infusion and proteinuria six weeks after doxorubicin injection showed a significant correlation. Moreover, a significant correlation was also found between renal blood flow at the time of injection and proteinuria 6 weeks thereafter. Secondly the group that received ANGII prior to the injection with doxorubicin did not develop less proteinuria than the saline treated group.

Conclusion: In conclusion, we extended the concept that baseline vessel tone predicts the susceptibility to renal damage by *in vivo* measurements of baseline glomerular diameter. Moreover, reduction in RBF by ANGII infusion during the infliction of renal damage does not limit development of kidney damage. The latter may signify that RAAS activation augments the damaging effect of toxic substances to the kidney.

Introduction

Susceptibility to renal injury varies largely among individuals both in the experimental and clinical setting[1-3]. This is exemplified by the existence of rat strains that are vulnerable or resistant to renal damage[4-6]. Even within one rat strain, challenges such as increased systemic blood pressure or subtotal nephrectomy[7] lead to highly variable renal damage, which cannot be explained by differences in damaging stimuli. Generally, the factors governing this individual susceptibility to renal damage are thought to be intrinsic to the kidney, and probably largely genetically determined[8;9]. This is supported by observations in experimental renal transplantation demonstrating that susceptibility to renal damage 'travels' with the kidney [10].

Recently, we found that the *in vitro* measured endothelial function of small renal arteries of healthy rats predicts the development of renal damage inflicted by subtotal nephrectomy[11], myocardial infarction[12], or doxorubicin-induced nephrosis. In the first two studies, individual rats with profound acetylcholine-induced endothelium-dependent relaxation developed less severe renal damage compared to those with compromised endothelial function. In the case of doxorubicin-induced nephrosis, this relationship was reverse, *i.e.*, rats with profound acetylcholine-induced endothelium-dependent relaxation developed more severe renal damage, possibly by larger exposure of the kidney to the cytotoxic drug. In a rat model of doxorubicin nephrosis, a single injection of the cytostatic agent doxorubicin leads to progressive renal damage with proteinuria, glomerulosclerosis and interstitial damage[13-15]. Remarkably, this relatively uniform challenge (standard doxorubicin injection), results in a largely variable renal damage amongst individuals[16].

Previous studies showing the relationship between healthy *interlobar* artery function and renal damage employed ex vivo isolated renal arteries. To explore whether this relationship holds true also at the resistance level of the afferent and efferent arteriole, we studied their diameter and responsiveness in *in vivo* experiments prior to induction of renal damage. Moreover, we studied whether renal damage as a result of the doxorubicin injection could be limited by co-infusion with a vasoconstrictor agent such as angiotensin II (ANGII).

Materials and methods

Experimental animals

Male Wistar rats (Hsd.Cpb.Wu (n=30); 200-250g; Harlan, Zeist, the Netherlands) were used, housed under standard conditions. The rats had free access to food and drinking water and received a normal diet (RMH-B, 2181; ABDiets, Woerden, the Netherlands). After arrival in our laboratory, the rats had an acclimatization period for one week to get used to their new environment. After this period the rats were trained for systolic blood pressure measurements, because stress would influence the measurements by elevating the blood pressure. All animal experiments were conducted in accord with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

Intravital microscopy

Figure 1 shows the experimental system used for the measurements. It consisted of a pencil video microscope with a corn shaped lens (optical magnification 3.5x) and a CCD camera (Nihon Kohden, Tokyo, Japan), a micromanipulator, a xenon light source (LB-18; Welch Allyn, Tokyo, Japan), a monitor (KLV-17HR1; Sony, Badhoevedorp, the Netherlands), a DVD recorder (RDR-GX7), and a computer for image analysis. The lens was fitted with a 12.7 mm greyscale CCD image sensor (XC ES55L; Toshiba, Tokyo, Japan) at the focal length (200 mm) of the lens). A green filter to complement red was placed in front of a CCD image sensor to enhance the contrast on the monitor between vessels and peripheral tissue. The CCD image sensor was connected to the camera module (DC700;Sony, Tokyo, Japan) and images were recorded as stacked image film (60 frames/min). The final spatial resolution of the video microscope was confirmed to be 0.86 mm with an electrical magnification of 520x. The scale of the captured video image was 752 x 582 pixels on the display, which allowed us to monitor only one glomerulus and its region in each experimental protocol.

Analysis of vascular diameters

Images at each measurement were captured as a stack of 60 frames using an image capture board (LG-3; Scion Computer Service, Frederick, Maryland, USA) installed on the image analysis computer. At least 3 clear frames, which were not influenced by respiration and heartbeat, were selected from the 60 frames in the captured stacks and analysed. To measure afferent and efferent diameters, image software (Scion Corporation, Frederick,

Maryland, USA) was used. Diameters were measured after calibration of the number of pixels (scale 2.42 pixels per μm).

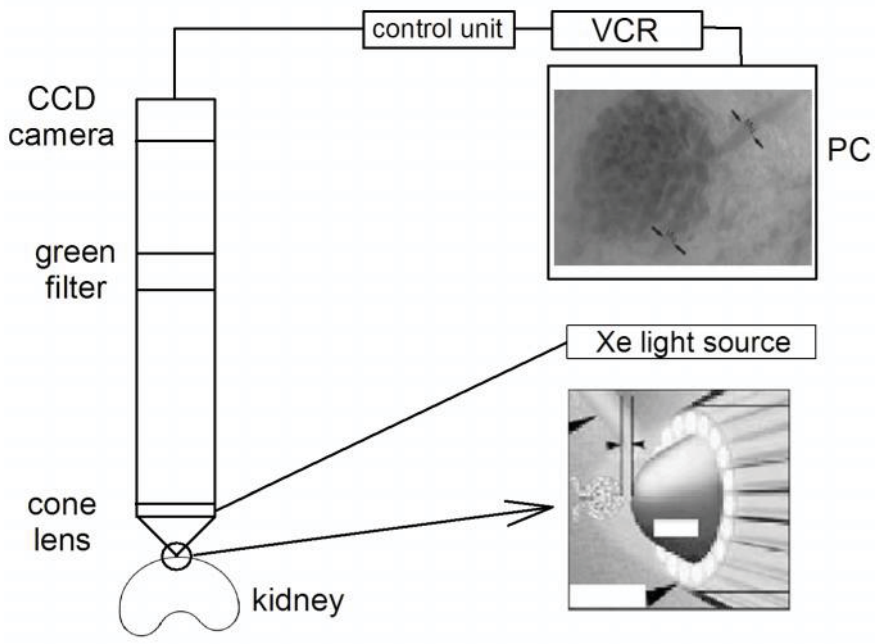

Figure 1. Schematic picture of experimental of intravital microscopy consist of pencil video microscope with corn shaped lens, micromanipulator, xenon light source, monitor a DVD recorder and computer for image analysis. Schematic picture adapted from Yamamoto et al [17]

Experimental protocol

The infusion protocol is shown in Figure 2. Rats were randomly divided into two groups: an experimental group, which received an angiotensin II infusion (n=16) during the administration of doxorubicin, and a control group which received a saline infusion (n=14). One day before the intravital experiment was performed, rats were individually housed. Before the experiment started, the rats were anesthetized with isoflurane/O_2 (2.5% isoflurane; Pharmachemie BV Haarlem, The Netherlands) to insert a shunt (BD Insyte-W 24 GA 0,75IN – 0,7x19 mm) in the tail vein. This shunt was used to administrate a bolus injection of sodium-pentobarbital (36 mg/kg; University Medical Centre Groningen Pharmacy, the Netherlands) after volatile anaesthetics were stopped and the animal started to show reflexes. If necessary, pentobarbital was re-administered to maintain a constant

level of anesthesia. During the experimental protocol the shunt was also used for bolus injection of doxorubicin. The operation region was anaesthetised with lidocain (20 mg/ml; Fresenius Kabi).

The carotid artery was cannulated for measurements of systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP) and heart rate (HR), which were recorded using a pressure transducer (Edwards Life sciences S.A., Saint-Prex, Switzerland) and an amplifier (model AP641G; Nihon Kohden, Tokyo, Japan). The jugular vein was cannulated for saline (control group) or angiotensin II (experimental group) infusion. To measure renal blood flow (RBF) and to carry out *in vivo* imaging of vascular diameter the abdomen was opened by midline incision. The left renal artery was exposed and an ultrasonic flow probe (model 1RB; Transonic Systems, Ithaca, New York, USA) was placed around it. RBF was continuously registered with a flow meter (model T106; Transonic Systems).

To expose glomeruli, the capsule of the renal cortex was removed, and a small slice of the renal surface was removed (maximum depth 0.5 mm) using a scalpel. The tip of the pencil-probe charge coupled device (CCD) video microscope was guided to the bottom of the excision. Superficial glomeruli in which the afferent and efferent arterioles could be visualized without affecting the blood flow were used for the experiment. One glomerulus per left kidney, in which the entire protocol could be completed, was monitored (Figure 1).

The protocol outline is presented in Figure 2. It consisted of a baseline of 10 minutes saline infusion. Then, angiotensin II infusion (30ng/kg/min; Bachem Bioscience) was started in the experimental group or the saline infusion continued in the control group. After 5 minutes, a doxorubicin injection (1.75 mg/kg; Doxorubin, Pharmachemie BV Haarlem, the Netherlands) was administered to both groups to induce renal damage. To determine acute effects of doxorubicin administration, the rats were monitored for 15 minutes following the bolus injection. After this period, the angiotensin II infusion was switched to saline, and both groups received saline infusion until the end of surgery.

Follow-up measurements of proteinuria and blood pressure

To evaluate the results of the protocol, proteinuria was measured in samples of 24-hours urine collected using metabolic cages on a weekly basis for 6 weeks. The urinary protein excretion was determined by nephelometry (Dade Behring III, The Netherlands). Weekly

systolic blood pressure measurements were carried out in conscious animals with tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA).

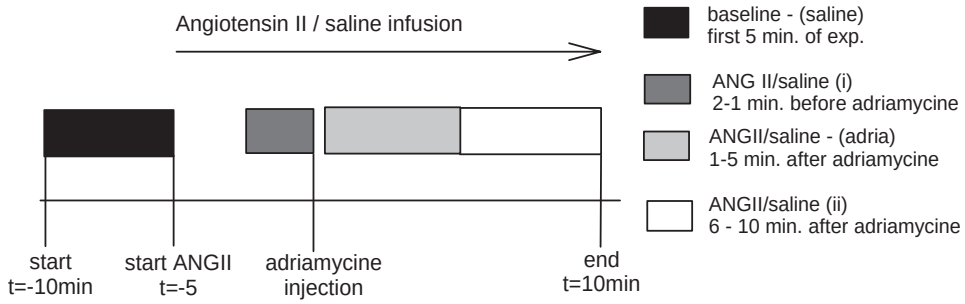

Figure 2. Schematic overview of the infusion protocol. Baseline measurements were taken when both groups received a saline infusion and was measured during the first 5 minutes of the experimental protocol. At time point "start ANGII infusion", the infusion of ANGII was started in the experimental group. During this period the control group continued with the saline infusion. Measurements of this period (ANGII/Saline i) were taken during the period 2 minutes before the injection of doxorubicin (adria). During time period "ANGII/Saline (adria)", the acute systemic effect of doxorubicin was measured. A few minutes before the end of the protocol, the longer effect of ANGII was measured (ANGII/Saline ii).

Statistical analysis

Results are expressed as mean ± SEM. Differences between the time periods were calculated and for comparisons a paired–sample t-test was performed (SPSS, Inc., Chicago, IL, USA). The relationships between measured parameters were calculated using Spearman's nonparametric correlation. Differences were considered significant at $p<0.05$.

Results

Acute changes of hemodynamic parameters and renal blood flow

Figure 3 shows the relative changes of the hemodynamic parameters at the different time points during the experimental protocol. To assess the ANGII-response in the experimental group, hemodynamic parameters were compared between baseline and the period before doxorubicin administration. ANGII infusion significantly increased systolic and diabolic blood pressure and reduced RBF (Figure 3), both compared to its own baseline ($p<0.05$) and to the control group ($p<0.001$). Heart rate was unaffected by ANGII infusion (Table 1, Figure 3). Injection of doxorubicin did not change any of these parameters either in ANGII or saline infused rats (Figure 3).

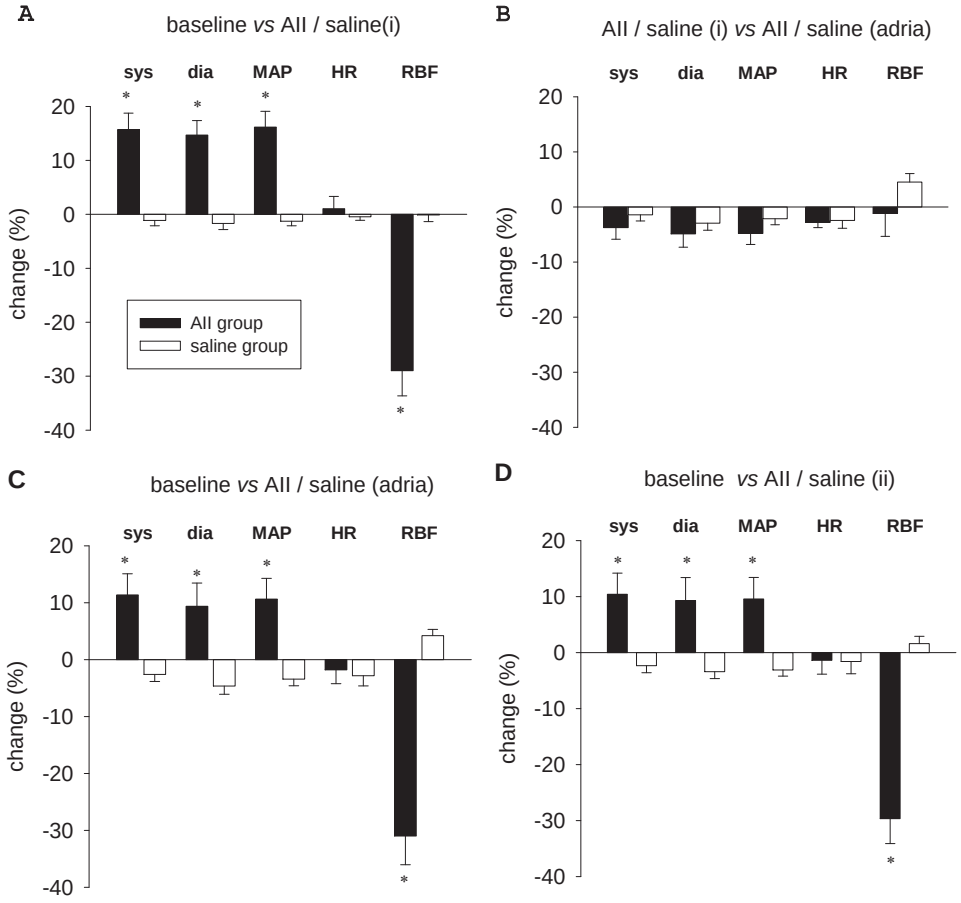

*Figure 3: Relative changes (%) of hemodynamic parameters between different time periods (as shown in figure 2). Relative changes between different points are shown for: baseline vs. the period before the injection of doxorubicin (adria; A) to show the effect of ANGII infusion only, the period before adria versus the period after adria (B) to show the acute effect of adria, baseline versus ANGII/Saline (i) to show the short effect of ANGII (C) and baseline versus ANGII/Saline (ii) to show the effect of longer ANGII infusion (D). Data are presented as mean ± SEM; * $p<0.05$.*

Table 1. Hemodynamic parameters during intravital microscopy. SBP- Systolic Blood Pressure (mmHg), DBP - Diastolic Blood Pressure (mmHg), MAP- Mean Arterial Pressure (mmHg), HR- Heart Rate (beats per minute, bpm), RBF- Renal Blood Flow (ml/min). Data are presented as mean ± SEM; [a] p<0.05 versus baseline of the same group; [b] p<0.05 versus control group at the same point

Experimental group (ANGII n=16)				
	Baseline	ANGII (before)	ANGII (adria)	ANGII (after)
SBP	137.1 ± 3.4	159.7 ± 6.1[a,b]	155.4 ± 4.7[a,b]	154.0 ± 4.7[a,b]
DBP	113.1 ± 3.2	130.3 ± 4.4[a,b]	126.4 ± 4.1[a,b]	126.0 ± 4.0[a,b]
MAP	125.0 ± 3.2	146.1 ± 5.0[a,b]	140.5 ± 4.0[a,b]	139.3 ± 3.9[a,b]
HR	374.6 ±11.3	379.3 ± 15.9	370.1 ± 15.3	372.0 ± 15.3
RBF	7.9 ± 0.5	5.3 ± 0.4[a,b]	5.0 ± 0.3[a,b]	5.1 ± 0.2[a,b]
Control group (Saline n=14)				
	Baseline	Saline (before)	Saline (adria)	Saline (after)
SBP	142.3 ± 3.8	140.8 ± 4.1	138.5 ± 3.8	138.9 ± 3.9
DBP	115.6 ± 4.1	113.5 ± 4.0	110.1 ± 4.0	111.4 ± 3.7
MAP	129.4 ± 3.8	127.6 ± 3.6	124.8 ± 3.6	125.2 ± 3.5
HR	345.3 ± 12.4	343.5 ± 12.1	335.7 ± 13.5	340.0 ± 13.6
RBF	7.3 ± 0.8	7.4 ± 0.8	7.6 ± 0.8	7.5 ± 0.8

Intravital microscopic analysis of afferent and efferent glomerular arterioles

To evaluate glomerular vascular function, changes in the diameter of afferent and efferent arterioles were assessed (Figure 4). ANGII infusion induced a significant contraction of both afferent and efferent arteriole, which was of similar magnitude (Figure 4A, B). There

was no significant change in diameter of glomerular arterioles in the control group during the same time period (Figure 4A, B).

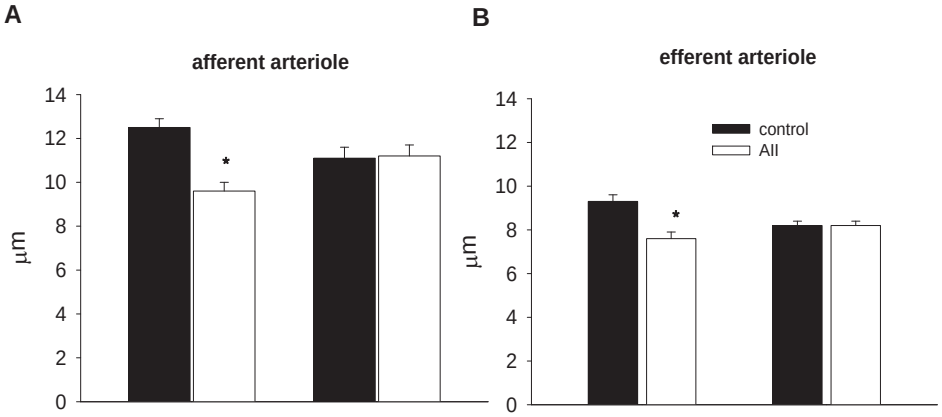

*Figure 4. Relative changes (%) in the A) afferent and B) efferent glomerular arteriolar diameter after saline (control group) and ANGII infusion. Data are expressed as mean ± S.E.M. *p<0.05*

To assess whether there was a relation between RBF and vessel width of either glomerular arteriole, correlation tests were performed. At baseline, a significant positive correlation was found between the diameter of the afferent glomerular vessel and RBF (Fig. 4C). In contrast, no correlation was found between RBF and efferent glomerular vessel diameter (Fig. 4D).

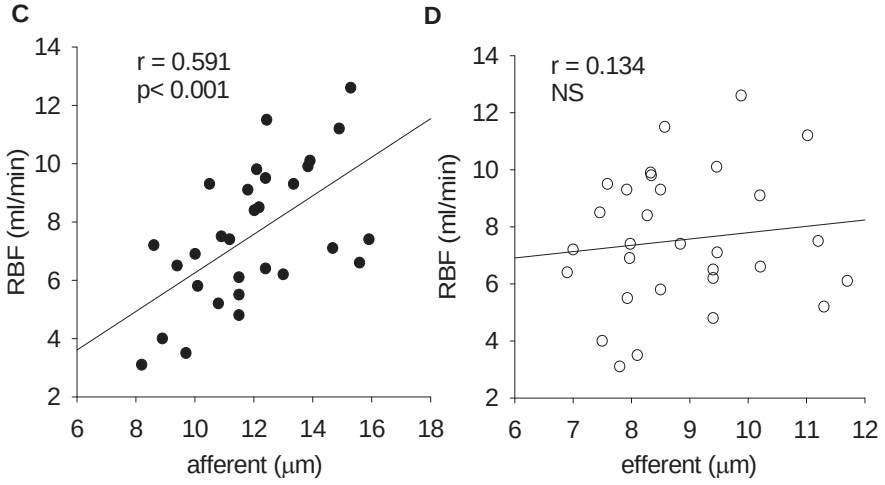

Figure 4. Relationship between basal RBF and basal diameter of C) afferent arteriole D) efferent arteriole.

Further, the effect of ANGII infusion on the relationship between arteriolar diameter, RBF and blood pressure was assessed. Constriction of afferent arteriole after ANGII infusion positively correlated with decrease of RBF (r = 0.576, p = 0.01, Figure 4.E), but no relationship was observed between efferent arteriole constriction and RBF (r = 0.211, p = NS, Figure 4.F). Furthermore, no relationship was observed between the change on afferent (r = 0.235, p = NS, Figure 4.G) or efferent arteriole (r = 0.162, p = NS, Figure 4.H) with change in SBP after ANGII infusion. Similarly, no significant correlations were found when absolute changes in RBF or vascular diameter were plotted against proteinuria (data not shown).

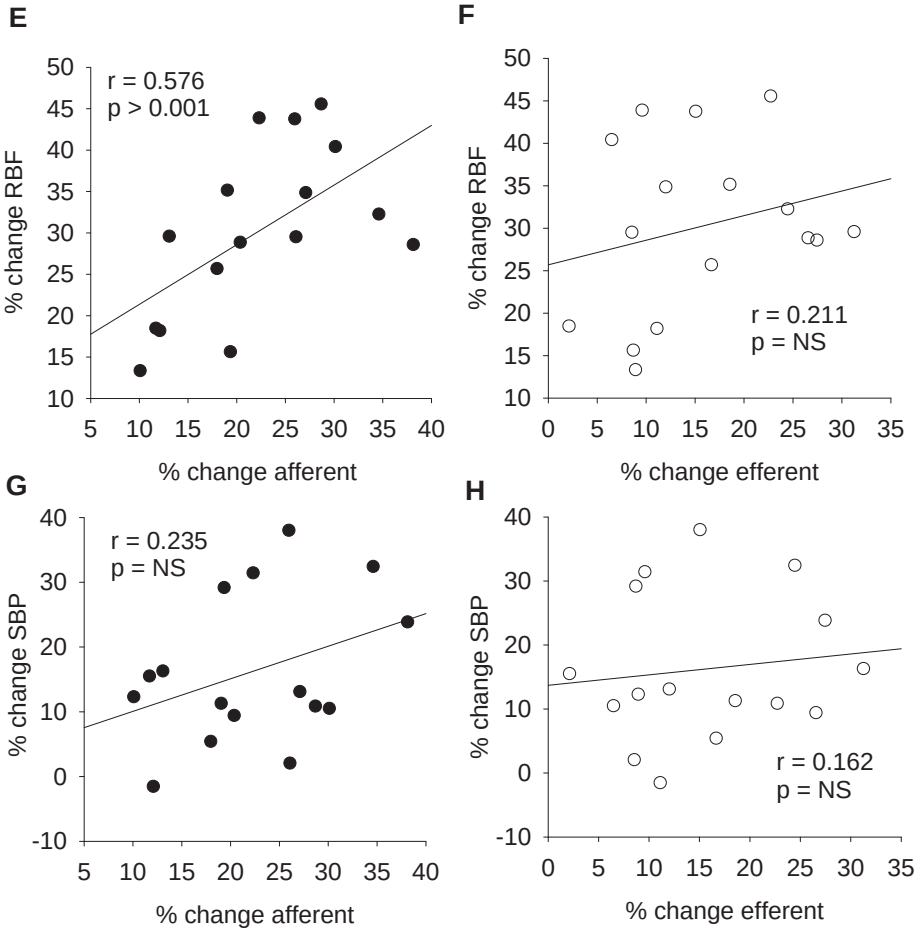

Figure 4.E) Relationship between change of RBF and change of afferent and F) efferent arteriole after ANGII infusion. G) Relationship between change of SBP and change of afferent and H) efferent arteriole after ANGII infusion.

Prediction of development of renal failure

As shown in Figure 5A, saline and ANGII infused animal groups gradually developed proteinuria after doxorubicin injection, and no statistical difference was found between ANGII infused and saline infused rats. Furthermore, there were no significant changes or differences in SBP in the saline and ANGII groups during the 6 weeks follow up (Fig. 5B).

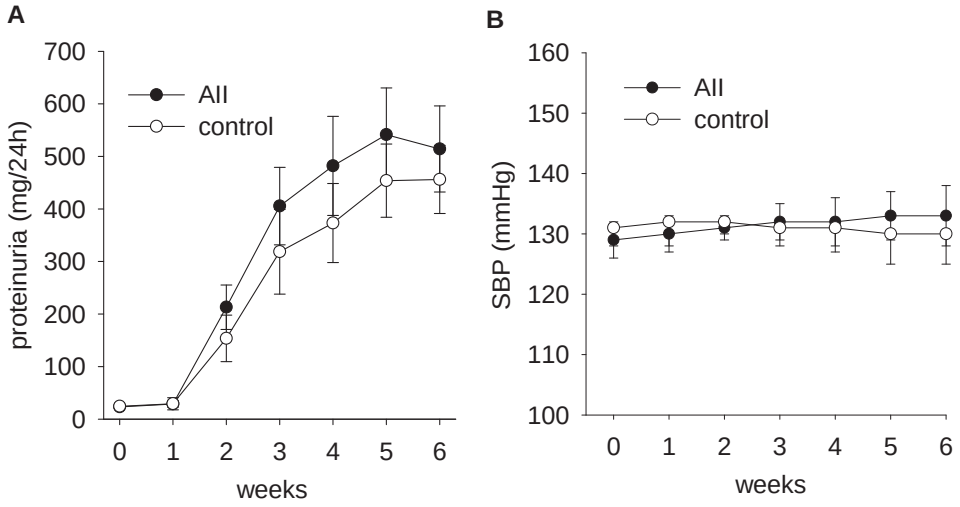

Figure 5A: Proteinuria and B) systolic blood pressure during 6 weeks follow up. Data are presented as mean ± SEM

To corroborate previous data indentifying that baseline RBF predicts development subsequent doxorubicin induced renal damage[18], a correlation was made of baseline RBF and proteinuria in saline infused animals (Figure 6A). Indeed, a positive correlation was found ($r = 0.580$, $p<0.05$). A similar analysis was performed in the group infused with ANGII during doxorubicin administration. Surprisingly, a positive correlation existed between the decrease of RBF upon ANGII infusion and proteinuria at week 6 ($r = 0.459$, $p = 0.05$). Similarly, a positive correlation was found between the reactivity of afferent arteriole to ANGII and proteinuria at 6 weeks (Figure 7A, $r = 0.538$, $p = 0.031$). In contrast, no relation was found between efferent arteriole and proteinuria at week 6 ($r = 0.09$, $p = NS$, Figure 7B). Similar correlations were obtained if area under the curve of proteinuria was used rather than proteinuria at week 6 (data not shown).

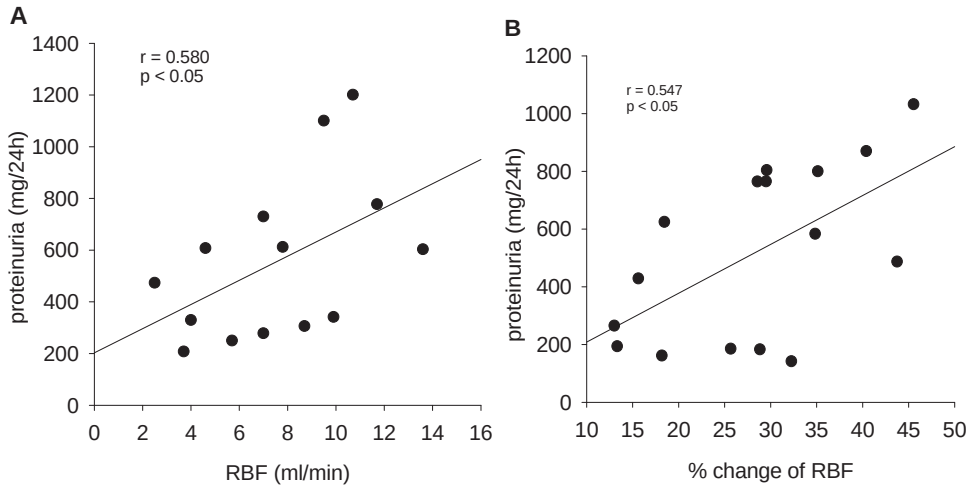

Figure 6A. Correlation analysis between individual values of basal renal blood flow (RBF) and proteinuria at week 6 in saline infused rats. Figure 6B Correlation analysis between individual values of change in renal blood flow (RBF) and proteinuria at week 6 in ANGII infused rats.

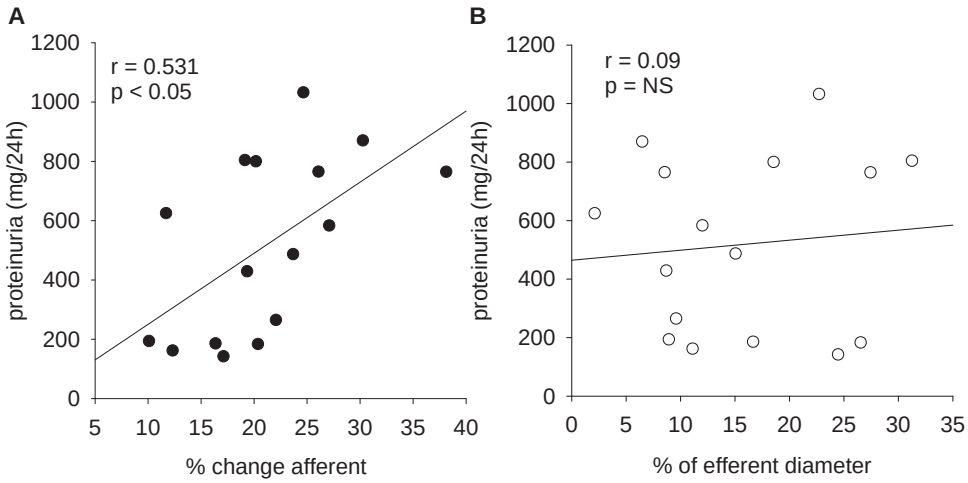

Figure 7. Correlation analysis between individual values of change in A) afferent, B) efferent arteriole and proteinuria in ANGII infused rats.

Discussion

This study shows that *in vivo* measurement of vascular performance of glomerular arteries predicts the development of proteinuria after doxorubicin-induced nephrosis. Moreover, we corroborate data that baseline RBF negatively correlates to development of proteinuria after subsequent administration of doxorubicin. Surprisingly, infusion of ANGII during the administration of doxorubicin, while substantially lowering RBF, did not limit the development of proteinuria in this model.

Infusion with ANGII induced a significant increase in all hemodynamic parameters except heart rate due to systemic vasoconstrictor effects[19]. While in the literature ANGII was reported to induce mainly vasoconstriction of the efferent arteriole[20;21], we did not observe differences between afferent and efferent arteries. This seems not due to an experimental artefact, as we found a consistent correlation between the diameter of the afferent arteriole and RBF, both at baseline and during ANGII infusion. In accord with our observations, Matsuda *et al.* found no differences in afferent and efferent arteriole contractility of canine superficial nephrons using the same visualising technique and approach as we did[22]. Nevertheless, they observed a larger respond to ANGII of efferent arteriole in (deeper) juxtamedullary nephrons. Thus, a possible explanation may be regional differences in response of glomerular vessels. Alternatively, the discrepancy between the observations may be explained by different experimental approaches, which may confound interpretation of the reactivity of glomerular arterioles. Nevertheless, the technique we used seems the less invasive compared to other techniques used to visualize glomerular vasculature.

The present study was performed to test the concept of predictive value of *in vivo* renal vascular function on the development of renal damage. Indeed, a positive correlation between baseline diameter of the afferent glomerular arteriole and the proteinuria was found. Moreover, afferent glomerular diameter inversely correlated to RBF. Thus our study confirms previous data showing that high baseline RBF correlated to excess renal damage. Further, our study suggests that vasotonus of the afferent arterioles is of importance to control RBF. Finally, our study extends the concept of predictive capacity of renal vascular function on renal damage to *in vivo* measurements, as previous from our laboratory showing a similar relation in doxorubicin nephrosis, 5/6 Nx and unilateral Nx with myocardial infarction employed interlobar arteries measured *ex vivo* [18;23;24].

A second aim of the study was to determine whether an ANGII induced increase in vascular diameter, and subsequent decrease in RBF, during doxorubicin infusion limits subsequent renal damage. Surprisingly, ANGII did not affect development of proteinuria, despite a substantial lowering of RBF during the infusion. These data are in contrast to experiments by *Ochodnicky et al.*, demonstrating a limitation of doxorubicin induced damage when animals were infused with L-NAME during induction of damage[18]. Notably, the reduction of RBF was similar in ANGII infusion (-32%) and L-NAME infusion (-22%). Thus, an insufficient reduction of RBF does not seem a plausible explanation for the lack of ANGII efficacy in limiting renal damage. Another possible explanation for the difference of effect of reduction RBF by ANGII and L-NAME might be the longer lasting RBF reduction after NO synthase inhibition, compared to the short-lived effects of ANGII infusion. However, as only a 12 min. obstruction of blood flow to the kidney following doxorubicin injection is needed to completely shield the kidney from doxorubicin induced damage[25], this explanation also seems unlikely. Thus, it seems that the beneficial effects of ANGII infusion in lowering RBF are offset by a negative effect of ANGII. Possibly, the generation of reactive oxygen species by ANGII induced activation of NADPH oxidase[26] amplifies the oxidative damage by which doxorubicin contributes to cellular damage[27]. Unfortunately, no further data are available on ANGII infusion or RAAS inhibition during doxorubicin injection.

Doxorubicin (Adriamycin) is a commonly used agent to treat cancers in humans. It has some serious adverse effects, especially organ toxicity, which in humans primarily manifests in the heart. The implication of our study is that should reduction of organ blood flow be employed to limit organ toxicity of doxorubicin chemotherapy, the vasoconstrictive agent should be chosen carefully.

In conclusion, we extended the concept that baseline vessel tone predicts the susceptibility to the infliction of renal damage to *in vivo* measurements of baseline glomerular diameter. Moreover, reduction in RBF by ANGII infusion during the infliction of renal damage does not limit development of kidney damage. The latter may signify that RAAS activation augments the damaging effect of toxic substances to the kidney.

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Chapter 4

Renal myogenic constriction protects the kidney from age-related hypertensive renal damage in the Fawn-Hooded rat

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(submitted)

Abstract

Introduction: To what extent age-related renal vascular dysfunction is involved in the development of renal damage is unknown. In the present study, we investigated the effect of ageing on renal vascular myogenic constriction (MC) in a genetic model of hypertension-associated renal damage, the Fawn-Hooded (FH) rat**.** FHL and FHH rats, which are without and with hypertension and renal damage at a young age, were compared after ageing to an age of 52 weeks.

Methods: Small renal arteries were isolated from 52 weeks old FHL and FHH rats, denuded and assessed for MC in a perfused vessel setup. Systolic blood pressure (SBP) and proteinuria were assessed every 10 weeks. Glomerulosclerosis and glomerular cross-sectional area were assessed after termination.

Results: Despite a similarly increased systolic BP in both strains at week 52 (166.0 ± 5.4 and 169.7 ± 5.4 mmHg in FHL and FHH, respectively), FHL rats had significantly lower proteinuria (48.9 ± 5.6 and 213.4 ± 20.7 mg/24h; $p<0.05$) and FGS (7.9 ± 1.5 and 43.5 ± 3.9%; $p<0.05$) compared to FHH rats. FHL renal arteries developed significantly higher MC compared to FHH (10.9 ± 1.4 and 5.1 ± 0.6 % of maximum MC over the whole pressure range; $p<0.05$). Cross-sectional area of glomeruli as representation of glomerular hypethrophy was significantly lower in FHL and inversely correlated with MC. Vascular responses to phenylephrine and MC in mesenteric arteries were similar in both strains.

Conclusion: Preservation of MC in renal arteries of the ageing FHL rat developing hypertension protects their kidneys from damage.

Introduction

Ageing is one of the most commonly found determinants of hypertension. When hypertension persists, functional and structural changes of several organs occur. In the kidney, which is highly sensitive to changes in systemic pressure, this may lead to renal damage.

Ageing is also associated with a decline in vascular function, which may be permissive in conveying hypertension associated end organ damage[1;2]. We recently established that renal vascular function in healthy animals predicts the renal damage after subsequent induction of kidney disease. These data showed that the better the endothelial dilatory function of small renal arteries, the less renal damage occurs in two renal damage models of hemodynamic origin, *i.e.*, the hypertensive 5/6th nephrectomy model[3] and the model of proteinuric cardio-renal interaction[4]. Thus, proper vascular function seems to play an essential role in protecting the kidney from damage in different models of renal failure.

Myogenic constriction (MC) is an important component of blood flow autoregulation and represents a mechanism regulating peripheral resistance. With an increase vascular transmural pressure, a contraction of vascular smooth muscle occurs, resulting in decreased vessel diameter and increased flow resistance. The precise mechanism of generation of MC is not yet fully known, but several pathways have been implicated[5-7]. Myogenic constriction is altered in several diseases. In rat, increased MC of mesenteric artery was found in chronic heart failure[8;9], while MC was reduced in animals with hypertension and chronic renal failure (CRF)[10]. Moreover, in humans, studies in small arteries isolated from gluteal fat biopsies from patients with type 2 diabetes mellitus show impaired MC[11]. In the kidney, MC serves to protect against transmission of high blood pressure into glomerulus, thus preventing glomerular hypertension. Renal interlobar arteries of the Fawn-Hooded Hypertensive (FHH) rat exhibit a diminished MC already at young age, preceding renal impairment and hypertension. Along with elevation of blood pressure in the FHH rat, renal damage starts to develop[12]. Taken together, these data suggest that preservation of vascular myogenic function of renal vessels in hypertension might be of prime importance to shield the kidney from glomerular changes and ultimately, CRF.

To explore whether renal damage in ageing associated hypertension is dependent on the integrity of MC of renal arteries, we studied 2 strains of the Fawn-Hooded rat. The Fawn-Hooded Hypertensive (FHH) rat is a genetic model of hypertension-associated renal failure, which develops systolic hypertension, proteinuria and glomerulosclerosis (FGS) already at a young age[13]. Previous studies showed that elevated glomerular pressure as a result of impaired renal autoregulation of the FHH rat[14] precedes the development of glomerular

injury[15-17], mainly due to impairment of renal MC. In contrast, the genetically similar Fawn-Hooded Low blood pressure (FHL) control strain does not develop hypertension or renal damage at a young age. Here we show that during ageing, both FH strains develop similar hypertension at 52 weeks of age. In FHL, MC was preserved and rats did not developed overt proteinuria or FGS. In contrast, FHH displayed impaired MC of renal arteries and development substantial proteinuria and FGS.

Materials and methods

Animals

Experiments were performed on 52 weeks old male FHL (n=8) and FHH (n=7) rats (FHH/Eur and FHL/Eur). Animals were obtained at an age of 9 weeks from Erasmus University Medical Centre (Rotterdam, The Netherlands) and housed under standard conditions at the animal facilities of the University of Groningen. Rats had free access to food and drinking water throughout the study. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

Blood pressure

Systolic blood pressure (SBP) was measured in trained, awake restrained animals by means of the tail-cuff method (IITC Inc, USA) as described previously[10;18].

Proteinuria

Urinary protein excretion was determined by nephelometry (Dade Behring III, The Netherlands) by placing the rats in metabolic cages for 24 h as described previously[10;18].

Clinical chemistry

Plasma and urine creatinine was measured by means of a photometric assay with the Jaffé method without deproteinization (DiaSys Diagnostic Systems, Holzheim, Germany) as described previously [10;18].

Renal histology

Glomerulosclerosis – PAS staining

Paraffin embedded kidneys were cut in 3 µm sections and stained with periodic acid Schiff (PAS) and the incidence of focal glomerulosclerosis (FGS) was microscopically evaluated according to standard procedures as described previously[19].

Glomerular morphometry – glomerular cross-sectional area

Glomerulal cross-sectional area was assessed on PAS stained sections. The cross-sectional areas were determined on 50 consecutive glomerular profiles per rat using computer-assisted morphometry. Only glomeruli without signs of sclerosis were included in the analysis.

Vascular measurements

Vascular reactivity of small renal and mesenteric resistance arteries

Small interlobar renal arteries and third-order branches of superior mesenteric arteries were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously [3]. Artery segments were cannulated on glass micropipettes and the vessel chamber was continuously recirculated with warmed (37°C) and oxygenated (5% CO_2 in O_2) Krebs solution with a pH of 7.4. An inverted light microscope attached to a video camera and video dimension analyzer was used to continuously register lumen diameter and thickness of the arterial wall.

Myogenic reactivity of small renal and mesenteric resistance arteries

Intraluminal pressure was set at 60 mmHg and arteries were allowed to equilibrate for 45 minutes and checked for smooth muscle viability by a single dose of phenylephrine (PE, $3x10^{-7}$ mol/L). To exclude role of endothelium in myogenic tone regulation, endothelium was removed by perfusing of arterial segment with 5 ml of air. Removal was confirmed by absence of dilative response to acetylcholine (ACh; $3x10^{-5}$ mol/L) following sub-maximal preconstriction with phenylephrine (PE, $3x10^{-7}$ mol/L). Following a wash out, intraluminal pressure was decreased to 20 mmHg and myogenic reactivity was studied by obtaining active pressure-diameter curves over a pressure range of 20-160 mmHg in steps of 20 mmHg. Each pressure step was maintained for 5 minutes to reach the stable contractile response. Thereafter, calcium containing Krebs solution was exchanged for calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether)tetraacetic acid

(EGTA, 2 mmol/l) and passive pressure-diameter curves were obtained over the same 20-160 mmHg pressure range.

Vascular reactivity of renal arteries to PE

To assess whether changed MC is not due to a generally lowered contractility of smooth muscle, vascular contractility of renal arteries was assessed by constructing dose-response curves by adding increasing dose of phenylephrine PE to recirculating bath ($3x10^{-5}$ – $1x10^{-8}$ mol/L) and intraluminal diameter was continuously recorded.

Solutions and drugs

Vessel segments were perfused with Krebs solution of the following composition (in mmol/L): NaCl 120.4, KCl 5.9, CaCl2 2.5, MgCl2 1.2, NaH2PO4 1.2, glucose 11.5, NaHCO3 25.0). All other compounds were purchased from Sigma (St. Louis, MO, USA).

Data analysis

Data are expressed as mean ± SEM. To characterize myogenic responsiveness, the following parameters were calculated from the pressure-diameter curve of each individual artery: Myogenic constriction was expressed as percent decrease in active diameter from the maximally dilated (passive) diameter determined at the same pressure in calcium-free/EGTA solution, *i.e.*, MC (%) = 100 [(DCa-free – DCa)/DCa-free], where D is the diameter in calcium-free (DCa-free) or calcium-containing (DCa) Krebs. For every individual artery, maximal myogenic tone of every segment was determined as the maximal value over the studied pressure range. Active tension in the vascular wall was calculated from measured active and passive vessel diameters using the following equation[20]:

T_a (dyne/cm) = - 1,333 (dyne/cm^2 x mmHg) x P x (DCa - DCa-free) x 10^{-4} (cm/µm).

Where T_a is the active wall tension (dyne/cm) and P the transmural pressure (mmHg).
Concentration-response curves to PE were expressed as percentage constriction of basal diameter. Areas under the curve (AUC) for MC were calculated from pressure curves and expressed as arbitrary units.

Statistical differences for vascular parameters, proteinuria, systolic blood pressure, creatinine, and glomerular cross-sectional area were determined by Student's independent t-test. The relationships between glomerular cross-sectional area, proteinuria and myogenic

constriction were calculated using Spearman's nonparametric correlation (Sigma plot, SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p<0.05$ (two-tailed).

Results

Systolic blood pressure – time course

Systolic blood pressure (SBP) was assessed at various ages in FHH and FHL, which is shown in Figs. 1A and 2A. At the age of 10 weeks, SBP values were not significantly different at 120 mmHg in FHH and FHL. Systolic blood pressure rose to a similar level at 52 weeks of age and did not differ among the FHH and FHL rats, reaching values of 169.7 ± 5.4 and 166.0 ± 5.4 mmHg for FHH and FHL, respectively (Fig. 1A, 2B). Thus, ageing induced hypertension in FHL. In between, the increase in FHH was steeper and blood pressure was significantly higher in FHH compared to FHL at 20, 30 and 40 weeks of age (Fig. 2A).

Proteinuria – time course

The time course of proteinuria is shown in Fig. 2B. At an age of 10 weeks, there was no significant difference in proteinuria between FHH and FHL. Thereafter, proteinuria rose steeply in FHH and was significantly higher compared with FHL at all ages including at 52 weeks, then reaching about 200 mg/24h in FHH compared with 45 mg/24h in FHL (Fig. 1B).

Renal damage

At 52 weeks, FHH rats had developed excessive renal failure compared to FHL, characterized by an increased plasma creatinine (34.0 ± 3.6 and 19.2 ± 0.9; $p<0.05$; Fig. 1C), increased proteinuria (213.4 ± 20.7 and 48.9 ± 5.6 mg/24h FHH *vs.* FHL, $p<0.05$; Figure 1B, 2B) and a significantly higher FGS (43.5 ± 3.9 and 7.9 ± 1.5 %; $p<0.05$; Fig. 1D) compared with FHL rats.

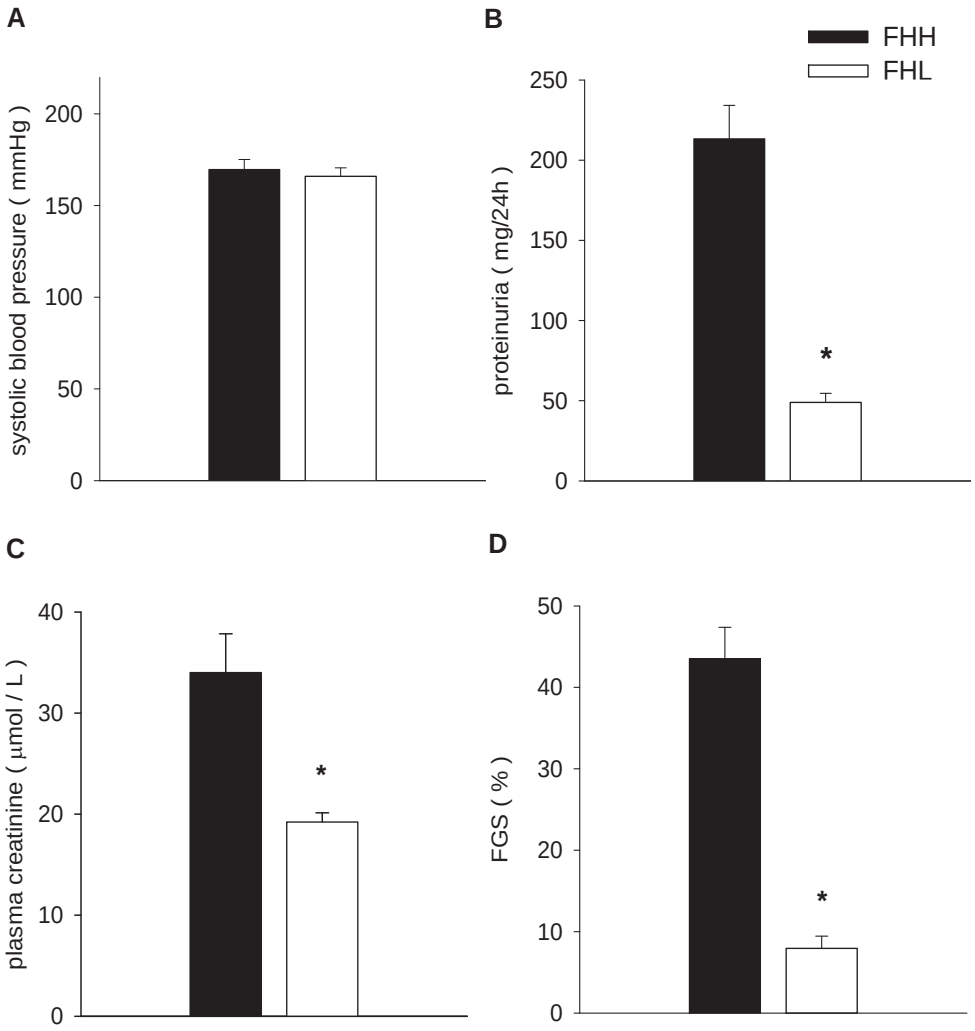

Figure 1. *Parameters of FH rats at 52 weeks of age.* ***A)*** *systolic blood pressure (mmHg) and* ***B)*** *proteinuria (mg/24h)* ***C)*** *plasma creatinine (µmol/L) and* ***D)*** *% incidence of focal glomerulosclerosis (FGS) studied on 52 weeks old FHH (black bars) and FHL (empty bars) rats.Data are expressed as mean ± S.E.M.. * p<0,05 compared with FHH.*

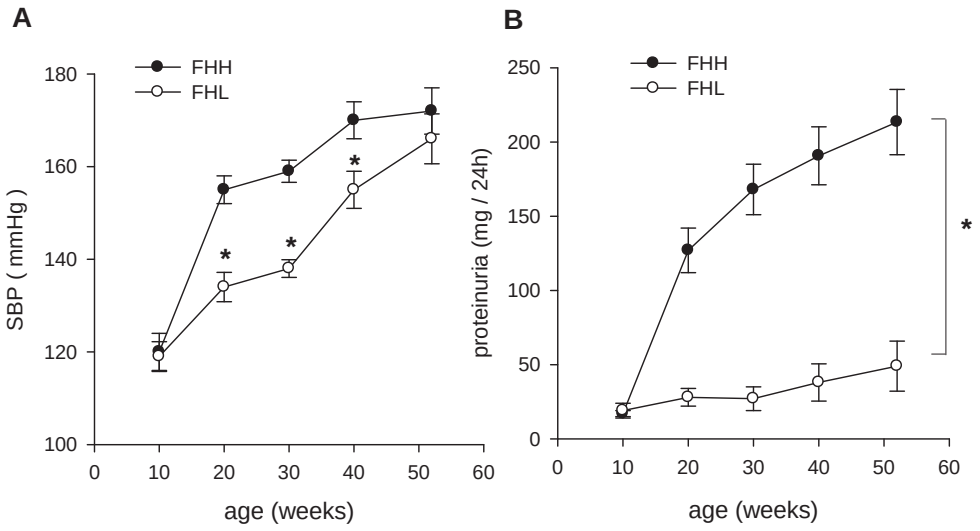

Figure 2. *Development in time of:* ***A)*** *SBP (mmHg) and* ***B)*** *proteinuria (mg/24 hours) during ageing in FHH and FHL rats. Data are expressed as mean ± S.E.M.. * p<0.01 compared with FHH.*

Renal artery myogenic constriction

At 52 weeks, pressure induced MC of small renal arteries was significantly higher in FHL (Fig. 3A and 3B) compared to FHH over a pressure range from 80-120 mmHg, reaching a maximal myogenic tone of 10.9 ± 1.4 and 5.1 ± 0.6 % of max. MC in FHL and FHH, respectively ($p<0.05$).

The comparison of the relationship between the change in active wall tension and intraluminal pressure in vessels obtained from kidneys of FHH and FHL is shown in Fig. 3B. Active wall tension (T_a) was significantly higher at pressure range 80-120 mmHg in FHL compared to FHH (T_a: 0.44 ± 0.05 and 0.16 ± 0.05 dyne/cm at 100 mmHg, respectively; $p<0.05$). Thus, at 52 weeks of age FHH demonstrates a stronger impairment of MC in the physiological range of intraluminal pressure than FHL.

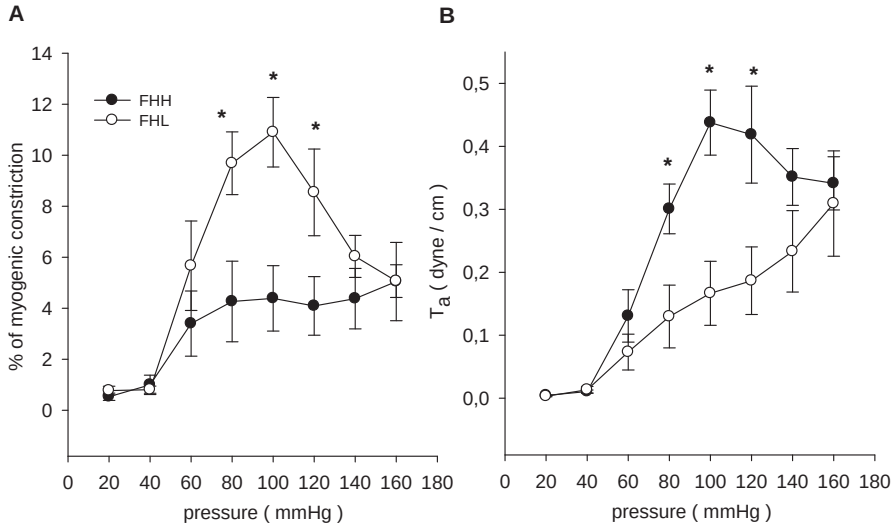

Figure 3. *Myogenic constriction in small interlobar arteries of FH rats.* ***A)*** *myogenic constriction expressed as % of passive diameter.* ***B)*** *T_a - active wall tension on studied interlobar arteries isolated from 52 weeks old FHL and FHH rats.Data are expressed as mean ± S.E.M. * p<0.05 compared with FHH.*

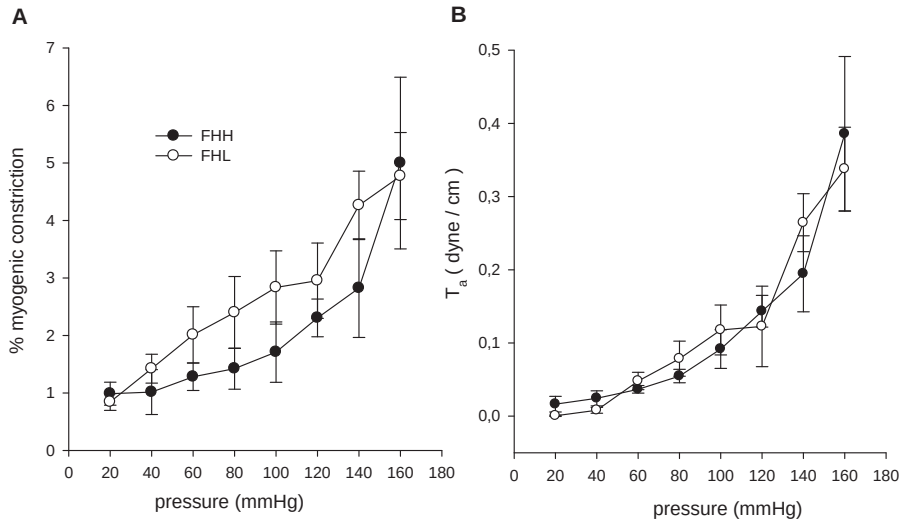

Figure 4. A) *myogenic tone expressed as % of passive diameter.* ***B)*** *T_a - active wall tension on studied mesenteric arteries isolated from 52 weeks old FHL and FHH rats. Data are expressed as mean ± S.E.M.. No significant difference were observed.*

Mesenteric artery myogenic constriction

In order to explore whether impaired MC is confined to the renal artery or a generalized phenomenon throughout the peripheral vasculature, MC was also assessed in mesenteric arteries of rats aged 52 weeks. Small mesenteric arteries developed MC dependent of the intraluminal pressure applied to the vessel. MC did not significantly differ between FHL and FHH over the whole pressure range (Fig. 4A, 5B). Active wall tension (T_a) of the studied mesenteric arteries did not significantly differ between the experimental groups (Fig. 4B) over the whole pressure range (T_a: 0.11 ± 0.03 and 0.09 ± 0.02 dyne/cm at 100 mmHg in FHL and FHH, respectively), highlighting the difference between the mesenteric and the renal artery.

Contractile properties of renal arteries to phenylephrine (PE)

To determine whether lowered myogenic contractility of renal arteries from FHH is due to generally lowered ability of arteries to constrict, dose-response curves for PE were constructed. As shown in Fig. 5, there was no significant difference between the groups, suggesting that impaired MC in FHH cannot be explained by the impairment of overall contractile ability of the artery.

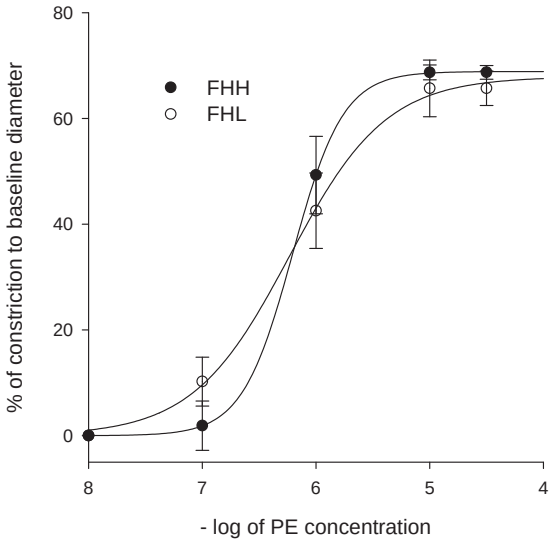

Figure 5. *Dose-response curve for phenylephrine (PE) obtained from interlobar arteries, isolated from 52 weeks old FHL and FHH rats. Data are expressed as mean ± S.E.M.. No significant difference observed*.

Glomerular morphometry

To determine whether hypertension affected glomerular structure (glomerular hypertrophy), we assessed cross-sectional area of glomeruli without FGS, as an early marker of glomerular injury that leads to sclerosis, in both FHL and FHH kidneys. As shown in Fig. 6A, the glomerular cross-sectional area in FHL kidneys was significantly smaller compared to FHH (12679 ± 388 and 16746 ± 875 μm^2; $p<0.01$), representing a 30% increase in cross-sectional area in FHH compared to FHL.

Correlation analysis

To confirm the protective role of MC in glomerular/renal protection, we analyzed the relationship between cross-sectional area of non-sclerotic glomeruli and MC (Fig. 6B). In FHL, a significant correlation was found of glomerular cross-sectional area with MC (inverse correlation: $r^2=0.5971$; $p<0.01$), whereas such correlation was absent in FHH.

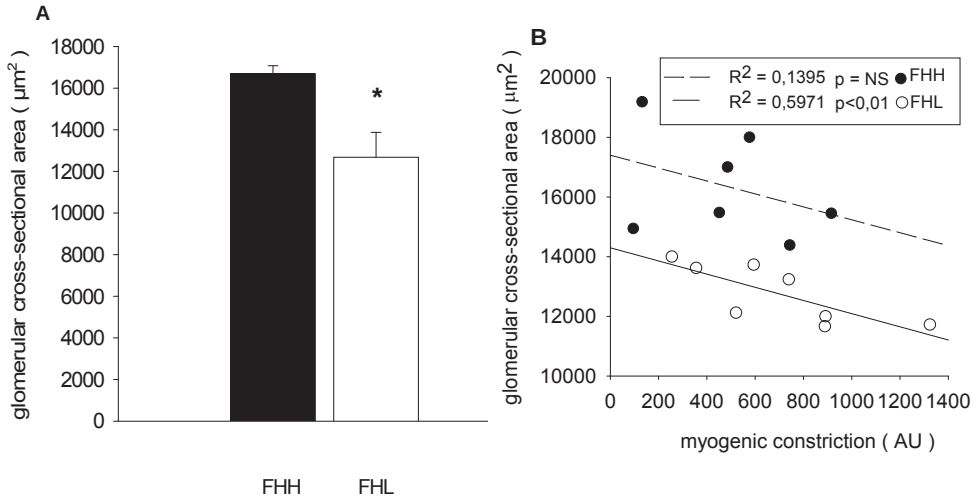

Figure 6. *Crossectional area of glomeruli and relation to myogenic constriction in 52 weeks old FH rats.* ***A).*** *The cross-sectional area (μm^2) of measured glomeruli from histologically processed kidney samples isolated from 52 weeks old FHL and FHH rats.* ***Figure 6B)*** *Correlation between individual values in 52 weeks old FHL and FHH rats of glomerular cross-sectional areas (μm^2) and MC expressed as AUC calculated from % of passive diameter. Data are expressed as mean ± S.E.M.. * $p<0,05$ compared with FHH.*

Discussion

The current study in the ageing Fawn-Hooded rat demonstrates that the FHL rat, which is normotensive at a young age, develops hypertension with age which is accompanied by significant urinary protein loss and FGS. Surprisingly, although of similar genetic make-up as the FHH rat, which develops hypertension and renal damage at a younger age, the FHL rat does not display renal damage when blood pressure rises. In addition, we found a significantly higher myogenic constriction (MC) of isolated renal arteries in FHL compared to FHH in the physiologic pressure range, whereas such difference was absent in mesenteric arteries. The preservation of renal MC most likely protected the FHL kidney from systemic increases in blood pressure being transferred to the glomerulus. Previously, it was shown that increased intraglomerular pressure due to impaired autoregulation leads to renal damage in the young FHH rat[21]. In the current study, we found support for this view by demonstrating that intact glomeruli of the FHL rat have a smaller cross-sectional area and that there is a significant inverse correlation of MC with glomerular cross-sectional area. We conclude that preservation of renal MC protects the aged FHL rat from the development of renal damage. In other words, a patent MC protects from development of renal damage when ageing related hypertension arises.

Intact myogenic tone protects from renal damage

Results from the current study suggest that preserved MC of intrarenal arteries protects the kidney from age related hypertensive damage in the FHL rat. The limited data available on blood pressure in the interlobar arteries[22] suggest that pressure in the renal artery of the rat is almost equal to mean arterial pressure. With a mean arterial pressure of FHL rats in our study at 122 ± 2 mmHg, it is conceivable that MC of FHL arteries is sufficient to protect glomeruli from elevated systemic pressure. In concordance with this is the observation that a limited increase in systolic blood pressure by pharmacological intervention to 160 mmHg in FHL does not induce renal damage, while at a further increase of systolic blood pressure to around 200 mmHg severe renal damage occurs[23]. Data from the spontaneous hypertensive rat (SHR) substantiate our hypothesis that a patent MC protects the kidney from hypertensive damage. It is well established that SHR rats develop very high systemic blood pressure without signs of renal damage, suggesting that preglomerular vessels exhibit abnormally high MC. Indeed, Ito *et. al.*, showed SHR afferent glomerular vasculature to display extremely high MC, protecting the SHR kidney from transferring the high systemic pressure into the glomerular capillary network despite high renal perfusion pressure[24]. In

contrast, Daneshtalab *et. al*. showed that the stroke prone SHR (SHRSP) rat displays loss of MC in cerebral flow autoregulation during hypertension, facilitating cerebral hyperperfusion and hemorrhage in SHRSP [25].

Nonetheless, the importance of MC in renal protection is obvious from the FHH rat, where a decline of renal MC precedes renal damage and only once hypertension occurs renal damage starts to develop. Collectively, these data strongly suggest the importance of renal MC in protection of the kidney against hypertension.

Intrinsic protective mechanism of renal vascular bed of FHL

It is clear from the present study that the impact of hypertension on MC differs among the studied vascular beds. While we observed preserved MC in the renal arterial bed in FHL, MC of mesenteric arterial segments did not differ between the two strains. Moreover, MC in mesenteric artery in aged rats was diminished in both strains when compared to younger age under similar conditions[12]. This might indicate that the renal vascular bed of FHL has an intrinsic mechanism protecting it from loss of MC in the presence of hypertension. The nature of MC is largely unknown; especially the mechanotransduction is still not yet fully explored. Several mechanosensitive ion channels, transient receptor potential channels (TRPC), integrins and other ion channels have been implied to play a role, and it seems that these mechanisms are specific for different vascular beds. The signalling of MC downstream of mechanotransduction share pathways with for instance the alpha adrenergic receptor (activation of PLC, IP3/DAG, PKC, Ca^{2+} sensitization)[7;26;27]. Because PE mediated constriction did not differ between FHL and FHH, it seems conceivable that a disruption in the mechanotransduction underlies the impaired MC in the renal vascular bed of FHH. Also, the decreased MC in FHH seems not due to a lowered general ability of arteries to constrict as evidenced by similar PE curves in both strains. Furthermore, we exclude the endothelium as a possible influence, since this layer was removed from the renal arteries studied. This suggests that the vascular smooth muscle component of vascular regulation is predominantly involved in protection of the FHL kidney against increases in intraglomerular pressure.

Myogenic constriction is related to glomerular surface area

Once failure of renal autoregulation in hypertension leads to glomerular hypertension, enlargement of glomeruli (glomerular hypertrophy) occurs as an early marker of renal failure onset[28;29]. Indeed, glomerular cross-sectional area of FHL kidneys was about 30% lower compared to FHH. The correlation observed between glomerular cross-sectional area,

MC and proteinuria further supports our hypothesis that a patent renal MC protects from transferring the systemic pressure into the kidney. The interlobar arteries used in this study are not the same as the glomerular afferent and efferent arteriole and their contribution to renal autoregulation and preglomerular resistance is thought less important. However, despite the relationship between loss of myogenic tone and glomerular enlargement, we cannot discriminate whether patent preglomerular artery function precludes glomerular damage, or that change in the interlobar artery reflects similar changes in glomerular arterioles.

Tubuloglomerular feedback

Since we did not examine tubuloglomerular feedback (TGF), we cannot exclude it as other possible (autoregulatory) mechanism that might have protected glomeruli in FHL rats. Nevertheless, in FHH rat TGF seem to be intact[30], therefore suggesting a minor role in renal damage in this particular model. Together with previous findings, this suggests that myogenic constriction plays a dominant role in renal protection against hypertension in the FH rat.

Blood pressure and the susceptibility to renal damage

Although only a minority of hypertensive patients develops CRF[31], African-Americans with hypertension have a 16-fold higher risk to develop renal failure than hypertensive patients of Caucasian decent[32], indicating that the susceptibility to develop CRF has a genetic background. The genetic background of CRF in FH rats has been partly elucidated; several quantitative trait loci (QTLs) have been linked to hypertension and CRF in FHH. Proteinuria QTLs, such as *Rf-1* through *Rf-5* have been shown to be involved in the pathogenesis of CRF, independent of systolic blood pressure QTLs (*Bpfh-1*, *Bpfh-2)*[33]. Thus FHH, displaying HT and renal failure susceptibility, represent a unique model of hypertension associated CRF. However, comparison of the FHH and FHL genotypes showed that markers flanking alleles of the *Rf-1* region were identical in both strains[23]. This indicates that the mutation in *Rf-1* gene is likely to be also present in the FHL strain. Consequently, challenging the FHL with an increase in blood pressure, either spontaneously (age; this paper) or induced (L-NAME[23]), is a relevant model of hypertension in the setting of susceptibility to renal failure.

Conclusion

In the present study, we show that despite age-related hypertension, FHL rat do not develop renal damage. The renal protection in FHL is most likely due to the preservation of myogenic constriction of renal arteries, thus limiting glomerular distention and damage. Taken together, we provide evidence for our hypothesis that impaired MC of the renal vasculature precedes and determines the development of renal damage.

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Chapter 5

Losartan protects mesenteric arteries from ROS associated decrease of myogenic constriction in 5/6 Nephrectomized rat

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Abstract

Background: Chronic renal failure (CRF) is associated with hypertension, proteinuria, loss of myogenic constriction (MC) of mesenteric arteries and increased production of reactive oxygen species (ROS) under experimental conditions. Our previous results showed, that ACE inhibitor therapy is effective in slowing down the progression. Therefore we wanted to extend this observation and test whether the inverse AT_1 receptor agonist losartan was effective in preventing loss of MC in a rat model of CRF and furthermore we wanted to test whether acute ROS scavengers could improve MC.

Methods: Rats were 5/6 nephrectomized (5/6 Nx) and treated for 12 weeks thereafter with vehicle or losartan (20mg/kg/day; 5/6Nx+LOS). Upon autopsy, MC of mesenteric arteries were measured in the presence/absence of tempol and catalase.

Results: Systolic blood pressure and proteinuria in 5/6Nx+LOS was significantly lower than in the 5/6Nx group. Moreover, MC of 5/6Nx+LOS arteries was significantly increased compared to the untreated 5/6Nx group (maximum of MC: 32.3 ± 6.9 *versus* 8.9 ± 3.8 % ($p<0.01$)). Tempol + catalase significantly increased MC in the untreated 5/6Nx group, but not in the 5/6Nx+LOS group (increase in MC: 59.7 ± 13.0 ($p<0.05$) *vs* 17.0 ± 15.1 %).
*Conclusion***:** These results support the role of RAAS and ROS in vascular muscle dysfunction of systemic vessels in CRF.

INTRODUCTION

Chronic kidney disease (CKD) is a worldwide health problem and associated with an increased risk of cardiovascular disease and chronic renal failure (CRF). In both clinical and experimental conditions, proteinuria(1) and glomerulosclerosis(2) are hallmarks of CKD leading to end organ failure. Pathological alterations of the vasculature, both in a functional and structural way(3) vary in intensity depending on the progression of CKD. The ability of small arteries to constrict with elevated transmural pressure, termed myogenic constriction (MC), varies in certain vascular beds. MC represents the main aspect of peripheral resistance, serves as an autoregulatory mechanism to protect organs from damage and maintains their constant blood supply. The exact mechanism of MC is not yet completely clarified and several pathways have been suggested to be involved(4;5)·(6) Moreover, MC varies among different arterial beds under different physiological(7) and/or pathological conditions(8)·(9).

Several animal strains spontaneously develop disturbances in MC. The spontaneously hypertensive rat (SHR) for instance develops excessive contractile responses in the renal(10) and peripheral vasculature(11), contributing to elevated peripheral resistance and high systemic blood pressure. Furthermore, the Fawn-Hooded Hypertensive (FHH) rat develops hypertension-associated renal damage as a result of an impairment in MC of the renal afferent vasculature(12), while peripheral vascular beds do not display these alterations(13).

The induction of organ damage such as chronic heart failure causes an increase in myogenic response of mesenteric arteries in the rat. In contrast, CRF induced by 5/6 nephrectomy (5/6Nx) causes loss of myogenic response in the same arterial bed. In both cases, treatment with ACE-inhibitors (ACE-i) reverses altered MC, implicating a role of the renin-angiotensin-aldosterone system (RAAS)(14;15) , possibly linked to a role of AT_1 receptors in the regulation of MC in the systemic vasculature.

Metabolism of oxygen by vascular smooth muscle cells (VSMCs) generates potentially harmful reactive oxygen species (ROS), including superoxide anion and hydroxyl radicals, as well as hydrogen peroxide. Under normal physiologic conditions, the extent and rate of oxidant formation is balanced by the rate of oxidant elimination by antioxidant enzymatic system, such as superoxide dismutase (SOD) and catalase. SOD converts superoxide

radicals to H_2O_2, which is in turn converted into water and oxygen by catalase(16). However, disturbed balance between ROS and antioxidants results in oxidative stress, which is the pathogenic substrate of vascular damage in various cardiovascular pathologies, including hypertension, endothelial dysfunction and atherosclerosis(17),(18). Also, increased superoxide production contributes to reduced NO bioavailability and hence results in endothelial dysfunction. Nevertheless, pathways of MC are not yet fully elucidated and therefore the role of ROS in MC also remains uncertain.

Several experimental data indicate that increased oxidative stress contributes to hypertension in 5/6Nx(19;20). Treatment with tempol, which has a catalytic role in superoxide dismutation(21) and thus performing a similar reaction as SOD, improves aortic endothelial function(22;23) of rats that underwent 5/6Nx. Nevertheless, the pathway by which ROS acutely or chronically contributes to diminished myogenic response in 5/6Nx is yet unknown.

The aim of the present study was to investigate the chronic effect of the inverse AT_1-receptor agonist losartan on changes in MC of small mesenteric arteries of rats with CRF induced by 5/6Nx. Blood pressure and proteinuria was monitored weekly during the study. Twelve weeks after 5/6 Nx, the experiment was terminated and segments of the third order branch of the mesenteric artery were examined to assess contractile response to pressure in a perfused vessel set-up. Moreover, to clarify the acute role of ROS in diminished myogenic response of mesenteric arteries, a combination of tempol and catalase was applied to the bath, in order to reach complete ROS degradation.

Materials and methods

Animals, surgery and in vivo measurements

Male Wistar rats (250 - 275 g, n=16) were obtained from Harlan (Zeist, The Netherlands) and housed under standard conditions at the animal facilities of the University of Groningen. Animals had free access to food and drinking water throughout the study. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen. By laparotomy and under anesthesia with 3% isoflurane in

N_2O/O_2 (2:1), rats underwent 5/6Nx by removing the right kidney and by ligating two or three branches of the renal artery of the left kidney, leading to infarction of approximately 2/3 of this kidney. Postoperatively, rats received a subcutaneous injection of diluted buprenorphin (Temgesic®; 0.01 mg/kg) for analgesic purposes. To achieve maximal therapeutical response, we administered losartan one week prior to 5/6Nx and therefore one week before surgery, rats were randomized (based on chance) in two groups: untreated (5/6Nx, n=8) and treated with losartan (5/6Nx + LOS, n=8). Losartan was dissolved in the drinking water at a dose of 20 mg/kg body weight/day.

Vascular reactivity of small mesenteric resistance arteries

Small third-order branches of superior mesenteric arteries were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously(24). Artery segments were cannulated on glass micropipettes and the vessel chamber was continuously recirculated with warmed (37°C) and oxygenated (5% CO_2 in O_2) Krebs solution with a pH of 7.4. An inverted light microscope attached to a video camera and video dimension analyzer was used to continuously register lumen diameter and thickness of arterial wall .

Myogenic reactivity of small mesenteric resistance arteries

Intraluminal pressure was set at 60 mmHg and arteries were allowed to equilibrate for 45 minutes and checked for smooth muscle and endothelium viability by a single dose of phenylephrine (PE, $3x10^{-7}$ mol/l) and acetylcholine (ACh; $3x10^{-5}$ mol/l), respectively. Following a wash out, intraluminal pressure was decreased to 20 mmHg and myogenic reactivity was studied by obtaining active pressure-diameter curves over a pressure range of 20-160 mmHg in steps of 20 mmHg. Each pressure step was maintained for 5 minutes to reach the stable contractile response. Thereafter, calcium containing Krebs solution was exchanged for calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether)tetraacetic acid (EGTA, 2 mmol/l) and passive pressure-diameter curves were obtained over the same 20-160 mmHg pressure range.

$Alpha_1$ receptor mediated contractility and endothelium-dependent relaxation of mesenteric arteries:

Following the determination of myogenic curves, intraluminal pressure was set back to 60 mmHg, arteries were washed and stabilized for 20 minutes. Thereafter, dose-response curves for phenylephrine (PE) were assed by adding cumulative dose of PE (10-8 – 10-

5mol/L) to the recirculating bath. Subsequently, after washout period arteries were pre-constricted with PE (10-6 mol/L). Endothelium-dependent relaxation was assessed by administering cumulative doses of ACh (10-9 - 10-5mol/L) to the recirculating bath in presence of indomethacin (10-5 mol/L) and indomethacin + L-NMMA (10-4mol/L) to investigate contribution of NO and EDHF to endothelium-mediated relaxation.

Involvement of ROS in myogenic reactivity and endothelium-dependent relaxation of small mesenteric resistance arteries

To investigate involvement of ROS in myogenic reactivity and endothelium dependent relaxation, on a separate arterial segments active pressure-diameter curves and ACh dose response curves were obtained as mentioned above in presence of tempol (100 μmol/l) and catalase (500 U/ml).

In vivo

Blood pressure

Systolic blood pressure (SBP) was measured weekly in awake restrained animals by means of the tail-cuff method (IITC Inc, USA). Twelve weeks after 5/6 Nx, the experiment was terminated and rats were sacrificed.

Proteinuria

Urinary protein excretion was determined by nephelometry (Dade Behring III, The Netherlands) weekly up to 12 weeks after 5/6 Nx by placing the rats in metabolic cages for 24 h.

Clinical chemistry

Plasma and urine creatinine was measured by means of a photometric assay with the Jaffé method without deproteinization (DiaSys Diagnostic Systems, Holzheim, Germany) and creatinine clearance was calculated as:

creatinine clearance = (urine creatinine x urine flow) / (plasma creatinine x bodyweight).

Renal histology

Paraffin embedded kidneys were cut in 3 μm sections and stained with periodic acid Schiff (PAS) and the incidence of focal glomerulosclerosis (FGS) was microscopically evaluated according to standard procedures as described previously(25).

Solutions and drugs

Vessel segments were perfused with Krebs solution of the following composition (in mmol/L): NaCl 120.4, KCl 5.9, CaCl2 2.5, MgCl2 1.2, NaH2PO4 1.2, glucose 11.5, NaHCO3 25.0). Losartan (Cozaar®) was purchased from Merck Sharp & Dohme BV (The Netherlands). All other compounds were purchased from Sigma (St. Louis, MO, USA).

Data analysis

Data are expressed as mean ± SEM; n values represent the number of investigated rats as well as the number of investigated arteries since one artery segment per rat was used for the same protocol. To characterize myogenic responsiveness, the following parameters were calculated from the pressure-diameter curve of each individual artery:

Myogenic tone, describing myogenic behavior of an artery at a given pressure, was expressed as percent decrease in active diameter from the maximally dilated (passive) diameter determined at the same pressure in calcium-free/EGTA solution, *i.e.*, MC (%) = 100 [(DCa-free – DCa)/DCa-free], where D is the diameter in calcium-free (DCa-free) or calcium-containing (DCa) Krebs. For every individual artery, segment maximal myogenic tone was determined as the maximal value over the studied pressure range. Area Under myogenic tone Curve (AUC) was determined (Sigma Plot, SPSS, Inc.) and expressed in arbitrary units.

Concentration-response curves to acetylcholine (ACh) were expressed as percentage of preconstriction to phenylephrine. Concentration-response curves to phenylephrine were expressed as percentage of constriction to basal diameter.

Statistical differences for vascular parameters, proteinuria, systolic blood pressure, creatinine, body and organ weights, water intake and urinary output were determined by Student's independent t-test. Differences in myogenic tone curves were tested using LSD post hoc multiple comparisons applied to ANOVA for repeated measures. Differences were considered significant at $p<0.05$ (two-tailed).

Results

Animals

Following 5/6Nx, 4 rats prematurely died because of uremia. Consequently, 12 rats completed the study and were eligible for the full protocol analysis at termination (5/6Nx, n=6; 5/6 Nx + LOS, n=6). Prior to surgery (week 0) baseline values of body weight, water intake, urinary output and proteinuria were similar in both experimental groups (Table 1).

Metabolic parameters

Water intake and urine output were significantly higher in the 5/6Nx group compared to the 5/6Nx + LOS group (Table 1).

Table 1. *Clinical characteristics of experimental animals (5/6 nephrectomized (5/6Nx) and 5/6 nephrectomized rats treated with losartan 20 mg/kg (5/6 Nx + LOS) measured before losartan treatment (week-1), before 5/6 Nx (week 0) and termination (week 12 after 5/6 Nx).Data are presented as mean* ± SEM

	5/6Nx	**5/6Nx + LOS**
Body Weight (g)		
week 0	345±8	346±6
week 12	449±1	496±1*
Water intake (ml/ 24h)		
week 0	27±3	23±3
week 12	65±7	44±5*
Urine output (ml/24h)		
week 0	10±1	12±2
week 12	41±6	26±3*
Plasma creatinine (µmol/L)		
week 12	95±16	53±4*
Creatinine clearance (ml/min/100g body weight)		
week 12	4.58±0.69	7.05±0.75*
Proteinuria (mg/24h)		
week 0	22±3	25±3
week 12	251±44	104±21*
Systolic blood pressure (mmHg)		
week -1	119±3	125±7
week 0	124±2	106±2*$^{\#}$
week 12	154±4	125±3*
Focal glomerulosclerosis (%)		
week 12	41.9±13.2	7.7±5.6*
Left ventricle weight (mg/g body weight)		
week 12	3.08±0.1	3.06±0.2
Wet kidney weight (mg/g body weight)		
week 12	5.9±0.2	4.1±0.3*

*p<0.05 *versus* 5/6Nx at the same time point
$^{\#}$p<0.05 5/6Nx + LOS *versus* week -1

Blood pressure

Tail-cuff blood pressure was similar at start of the study (week-1),but was significantly lower before 5/6 Nx in the 5/6 Nx + LOS group (week 0) due to the treatment with losartan. Furthermore, SBP in the 5/6Nx + LOS group was significantly decreased compared to untreated 5/6Nx group from week 2 until the end of the study (week 12) (Figure 1A; Table1).

1.A

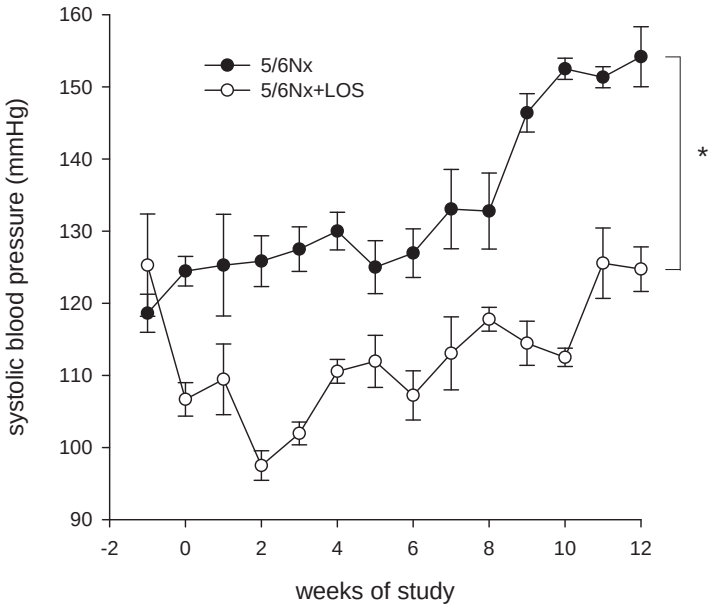

Figure 1A. *The development of systolic blood pressure (mmHg) in time before and after the subtotal nephrectomy. 5/6Nx - 5/6 nephrectomized and 5/6NX+LOS- 5/6 nephrectomized treated with losartan (20 mg/kg).Data are expressed as mean ±S.E.M. * p<0.01 compared with 5/6Nx*

Renal parameters

Within 12 weeks after 5/6Nx, animals developed renal failure characterized by increased proteinuria (Figure 1B), increased plasma creatinine, decreased creatinine clearance, higher kidney/body weight ratio and significantly higher focal glomerulosclerosis score in the 5/6 Nx group compared to the 5/6 Nx + LOS group (Table 1). Thus, losartan effectively counteracted development of renal damage and decline of renal function in 5/6Nx rats.

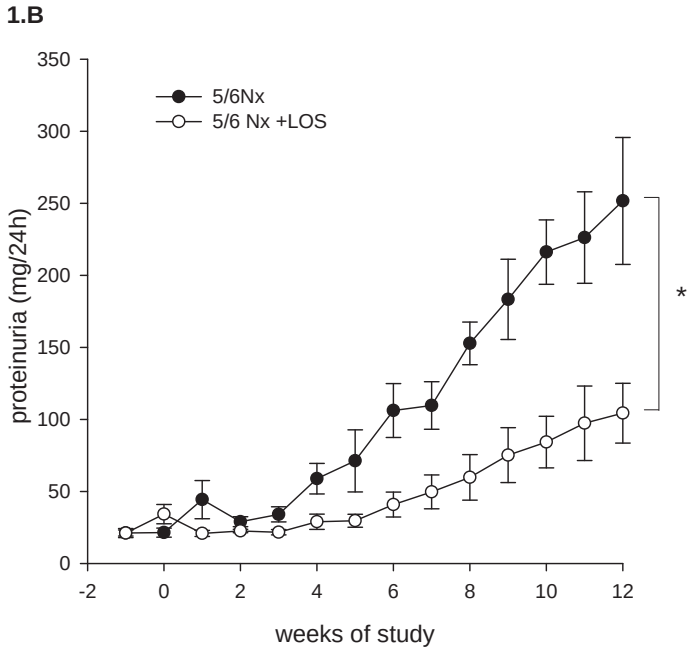

Figure B1. *The development of proteinuria (mg/24h) in time before and after the subtotal nephrectomy. 5/6Nx - 5/6 nephrectomized and 5/6NX+LOS- 5/6 nephrectomized treated with losartan (20 mg/kg).Data are expressed as mean ±S.E.M. * p<0.01 compared with 5/6Nx*

Myogenic constriction

As shown in Figure 2A, the passive diameters of small mesenteric arteries did not differ between the experimental groups over the whole pressure range, suggesting no apparent structural changes hampering maximal relaxant ability of the investigated arteries. Small mesenteric arteries developed myogenic tone dependent on the intraluminal pressure applied to the vessel.

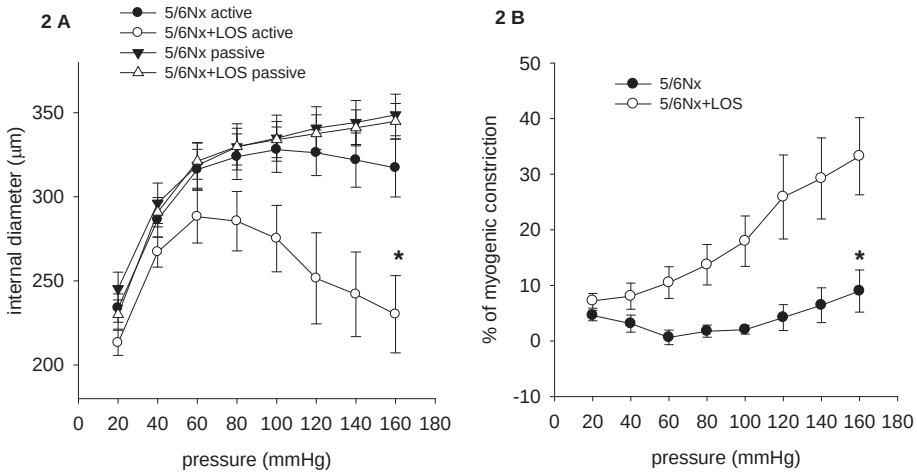

Figure 2. A) *Diameters of mesenteric arteries in response to stepwise increase of intraluminal pressure in the presence (circles, active tone) or absence (triangles, passive tone) of extracellular calcium.* ***B)*** *myogenic tone expressed as % of passive diameter. Studied in (5/6 Nx) and 5/6 Nx rats treated with losartan 20 mg/kg (5/6 Nx + LOS) 12 weeks after the operation. * p<0,05 compared with 5/6 Nx*

Active diameters were significantly higher in the 5/6Nx group over the pressure range of 100-160 mmHg compared with the 5/6Nx + LOS (Figure 2A; Table 2) group. Consequently, MC expressed as a percent of passive diameters was significantly higher in the 5/6Nx + LOS group compared to 5/6Nx over a pressure range 60-160 mmHg, reaching maximal myogenic tone of 32.3 ± 7 % and 8.9 ± 4 % in the 5/6Nx + LOS and 5/6 Nx group, respectively ($p<0.01$).

Involvement of ROS in diminished myogenic response

To investigate involvement of reactive oxygen species, vascular segments were incubated with tempol and catalase (TC) *in vitro.* Acute treatment with TC did not change the passive diameter of small mesenteric arteries of the groups over the whole pressure range (data not shown). As shown in Figure 2C, TC induced a significant increase in myogenic constriction of arterial segments from untreated 5/6 Nx rats (maximal MC: 8.9 ± 4 % in the 5/6 Nx *vs* 21 ± 4 % in the 5/6Nx + TC ($p<0.05$)). In contrast, TC induced a modest, non-significant

increase in the myogenic constriction of 5/6Nx + LOS group. Consequently, the TC induced increase in area under curve was significantly larger in the 5/6Nx group compared with the 5/6Nx + LOS (Table 2, Figure 2D) group.

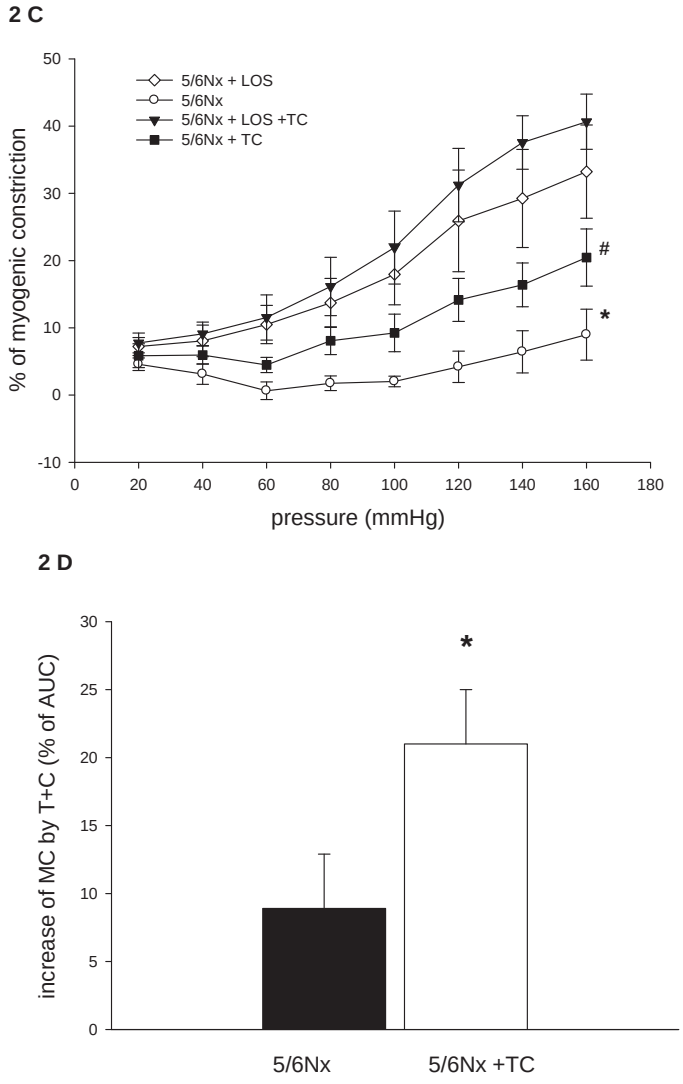

Figure 2C *myogenic tone expressed as % of passive diameter of mesenteric arteries in presence or without of tempol (100 µmol/l) and catalase (500 U/ml). TC indicates tempol + catalase.* ***D)*** *change of MC after incubation with T+C expressed as percentage of MC in the absence of TC. * p<0,05 compared with 5/6 Nx. # p<0,05 compared with 5/6 Nx+TC.*

Table 2. *Characteristics of myogenic reactivity of mesenteric arteries isolated from 5/6 nephrectomized (5/6Nx) treated or untreated with losartan 20 mg/kg/day (5/6 Nx + LOS). Max MC (%) means maximum of myogenic constriction over the whole pressure range, expressed as a percent of passive diameter. TC indicates (+) presence or (-) absence of tempol (100 µmol/l) and catalase (500 U/ml) in perfused Krebs solution. Data are presented as mean* ± SEM

	5/6 Nx		**5/6 Nx + LOS**	
TC	**-**	**+**	**-**	**+**
Max MC	8,9 ± 3,8	20,5 ± 4.2*	33,2 ± 6.9*	40,7 ± 4.1

*p<0.05 *versus* 5/6Nx without tempol + catalase (-TC)

Involvement of endothelium on MC and effect of ROS scavengers

To explore how is endothelium involved in impaired MC we decided to construct relaxation dose-response curves for acetylcholine in absence or presence of tempol and catalase, in both NO and EDHF level.

The mesenteric arteries isolated from the experimental animals responded to acetylcholine to a variable extent, however there was no significant difference observed between the studied groups in both NO (Figure 3A) and EDHF level as shown in and Figure 3C. Even more no significant difference was observed in presence of tempol and catalase in both NO (Figure 3B) and EDHF-mediated level (Figure 3). The endothelium-dependent relaxation characterized by the Area Under the acetylcholine Curve (AUC) in presence of indomethacin was 193.8±31.5 for 5/6Nx and 195.1±24.5 for 5/6Nx+LOS. The endothelium-dependent relaxation in presence of indomethacin and combination of tempol and catalase was 197.8±29.3 for 5/6Nx and 202.1±15.1 for 5/6Nx+LOS. Relaxation of mesenteric arteries characterized by AUC in presence of indomethacin and L-NMMA was 155.5±17.3 for 5/6Nx and 151.8±16.9 for 5/6Nx+LOS. Incubation of arterial segments with tempol and catalase did not significantly alter responses to acetylcholine in presence of indomethacin and L-NMMA. AUC of relaxation curves was 174.8±26.8 for 5/6Nx and 145.5±22.1 for 5/6Nx+LOS.

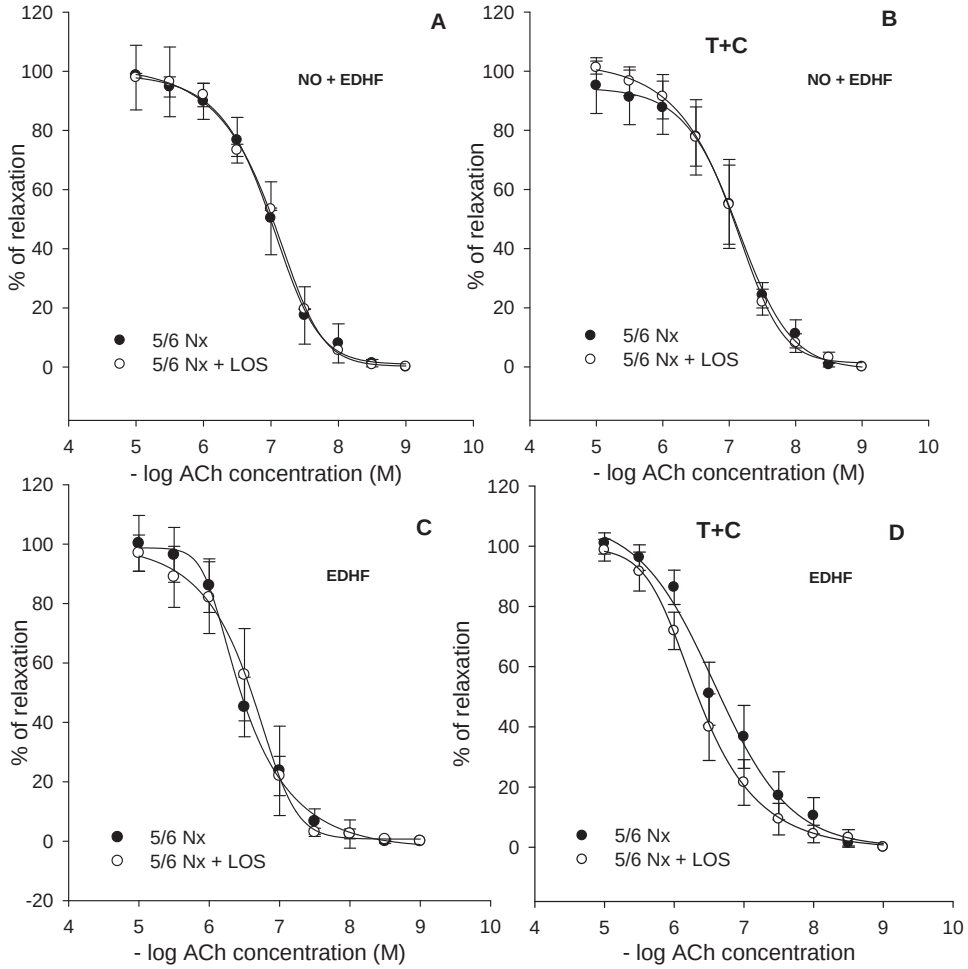

Figure 3. *Acetylcholine-mediated endothelium dependent relaxation curves of mesenteric arteries obtained from 5/6Nx and 5/6Nx+LOS rats in a presence of* ***A)*** *indomethacin,* ***B)*** *indomethacin and tempol + catalase,* ***C)*** *indomethacin + L-NMMA,* ***D)*** *indomethacin + L-NMMA and tempol + catalase.Data are expressed as mean ± S.E.M.. No significant difference observed.*

Discussion

Results of the present study indicate for the first time that chronic treatment with an angiotensin II (AII) - antagonist improves MC of mesenteric arteries of rats after 5/6Nx and furthermore we showed that ROS are acutely involved in it. Vettoretti *et. Al.* previously showed that chronic treatment with an ACE-i in the same setting protected mesenteric arteries from loss of MC(26). It is now shown that blockade of AT_1 receptors with losartan is also effective, confirming a role of AT_1 receptors in pathogenesis of renal failure associated loss of myogenic function in mesenteric arteries after 5/6Nx.

Systolic blood pressure, proteinuria

Treatment with losartan prevented an increase in systolic blood pressure (SBP) and proteinuria, which is in accordance with other studies(27), confirming the damaging effect of AT_1 receptor-mediated AII. There is an increase of SBP in the losartan group at 12 weeks after 5/6Nx compared to SBP before 5/6Nx suggesting progression in development of hypertension despite administration of losartan. This may be explained by other mechanisms involved in the pathogenesis after 5/6Nx, such as activation of the sympathetic nervous system(28).

Renal function, focal glomerulosclerosis

Animals treated with losartan showed improved renal function as evidenced by an improved creatinine clearance denoting hyperfiltration. We also observed a decreased incidence of focal glomerulosclerosis in rats treated with losartan, which confirms findings in previous studies(29;30). Part of the effect of losartan may have been caused by lowering blood pressure to (near) physiological levels. Alternatively, a direct role of AT_1 receptors on filtration apart from blood pressure lowering may contribute to preservation of renal function, since chronic AT_1 receptor antagonism also preserves renal function in animal models with normal blood pressure (31).

Myogenic constriction

The nature of the decreased MC in 5/6Nx is currently unknown. Previous studies showed a decrease in myogenic response of rat mesenteric artery after 5/6Nx(26) and this data suggests that increased blood pressure is not caused by elevated peripheral resistance, but increased blood pressure might be the driving force of diminished response of mesenteric arteries to pressure.

The current study confirmed our previous results, where 5/6 Nx decreases MC of mesenteric arteries and RAAS intervention reverses this phenomenon. However, Savage et. Al reported increased MC of the femoral artery after 5/6 Nx(32), which seems to be contradictory to the current results. However, differences between vascular beds and the used model showing no hypertension might explain this difference.
There is lack of data regarding MC of mesenteric artery in the 5/6 Nx model. Several questions arise when trying to explain decreased MC in this model. The general concept that hypertension is caused by increased peripheral resistance due to increased MC of peripheral resistance arteries seems to fail in 5/6 Nx where MC was found to be diminished. Whether hypertension and/or impaired renal function affects MC remains unclear. With lowering blood pressure comes improvement of deteriorated renal function in 5/6 Nx thus is difficult to distinguish between this two possible causes.

Moreover, we found that nephrotoxic drugs such as adriamycine cause renal failure without presence of increased blood pressure and impairs MC of mesenteric arteries. Based on this fact we hypothesized, that worsening of renal function is responsible for impaired MC of mesenteric arteries [Ochodnicky, Vavrinec, 2008; not published]. Nevertheless, Murata et. al. have shown that adriamycin directly decreases contractility of cultured rabbit mesenteric arteries and is even more able to cause ROS associated downregulation of the alpha-1 receptor(33).
Up to our best knowledge there is currently no evidence available of blood pressure lowering regimes in 5/6 Nx and its effect on MC of the mesenteric artery. The same holds for regimes that might improve renal function only without lowering blood pressure. However, further exploration of diminished MC of the mesenteric artery after 5/6 Nx should be focused on distinguishing between these options.
Chronic treatment with ACE-i is beneficial after 5/6Nx; it decreases blood pressure, improves renal function and protects mesenteric arteries from loss of MC, confirming a role of the RAAS in renal damage but also revealing its action on peripheral vasculature(26). It is known that the myogenic response, which exact mechanism is so far not fully explored, shares similar pathways (GPCR, PLC, PKC, RhoA/Rho kinase)(34-36) with vasoconstrictive agents such as noradrenalin, endothelin, and AII. These pathways, which are activated during hypertension, have been implicated to strongly potentiate myogenic responses. Indeed, we showed involvement of activated AT_1 receptors in MC in models of chronic heart failure(37;38). Increased myogenic responses after induction of myocardial infarction were observed and acute antagonism of AT_1 receptor normalized excessively

increased myogenic response of mesenteric arteries. Conversely, downregulation of certain receptor types or pathways because of sustained activation, may theoretically decrease myogenic responses. Chronic treatment with losartan might have preserved AT_1 receptors from downregulation thereby preserving mesenteric arteries from decrease of MC caused by 5/6Nx.

Furthermore, we wanted to investigate whether decreased myogenic contractility is due to generally lowered contractile ability of vascular smooth muscle, due to a shifting from contractile to proliferative phenotype. Construction of dose-response curves for PE did not show any differences between the studied groups and even more TC had no effect on it (data not shown). Based on these results, we conclude that decreased MC of mesenteric arteries in 5/6 Nx group was not due to lower contractility of smooth muscle when compared to losartan treated animals. Hypertension and/or impaired renal function selectively decrease MC in 5/6 Nx rats, not affecting smooth muscle contractility in mesenteric artery.

Hypertension is known to cause arterial remodeling both on the macrovascular and microvascular level causing changes in vascular function. In the present study, we did not observe significant differences in wall thickness between both (data not shown). Moreover, there was no difference observed between passive diameters over the whole pressure range in-between the groups. Based on this, we conclude that arterial remodeling couldn't have affected MC.

Effect of tempol and catalase on MC

Oxidative stress is an important contributor to the development of vascular dysfunction found in various pathological conditions. Several experimental data indicate that increased oxidative stress contributes to hypertension in 5/6Nx(19;20). In the present study, involvement of oxygen superoxide and hydrogen peroxide in pathological decrease of MC is suggested by improved the myogenic response of mesenteric arteries after 5/6Nx by the combination of tempol and catalase. Support from other studies comes from the observation that peroxynitrite, that is formed by reaction of superoxide with NO, is known to inhibit myogenic response of rat posterior cerebral artery(39), suggesting direct involvement of ROS products in inhibition of MC. Nevertheless, studies with SOD knockout mice showed enhanced MC of mesenteric arteries from these animals, implicating that increased superoxide production in vascular tissue increases the contractile response of vascular smooth muscle to pressure(40). These findings might suggest that involvement of oxidative stress in MC is dependent on the specific disease model and/or vascular bed.

It is known that AII causes production of ROS via activating AT_1 receptors(41), which contributes to a large extent to the oxidative stress in 5/6Nx. Based on the fact that effect of ROS scavenging on MC in our experiment was much higher in untreated animals compared to those treated with losartan, we conclude that there is a direct involvement of ROS in decreased MC. The molecular mechanism still needs to be elucidated. However, in untreated 5/6Nx animals, tempol and catalase did not completely reverse myogenic response to the level of losartan treated animals. Therefore, acute ROS production at the time of testing in the organ bath does not seem to entirely govern diminished myogenic response of mesenteric artery in the 5/6Nx model.

Source of ROS in mesenteric artery

Recently, hydrogen peroxide was recently implied as source of EDHF(42). Therefore, one might hypothesize that the presence of hydrogen peroxide in vascular tissue due to increased ROS production after 5/6 Nx might be the cause of the observed decreased in MC. However, we can exclude this hypothesis because there was no difference in EDHF mediated relaxation in the present study. Moreover, there was no effect of catalase (which breaks down hydrogen peroxide) on vascular relaxation.

The endothelium as source of ROS affecting MC can be excluded in the present study. There was no apparent effect of tempol and catalase on relaxation, where ROS is largely involved in impairment of the NO pathway.

Moreover, we did not observe changes in endothelium mediated relaxation between studied groups, nor on nitrous oxide (NO) or endothelium derived hyperpolarizing factor (EDHF) level. Using the same model, Vettoretti *et. al.* showed impaired EDHF mediated relaxation of the mesenteric artery and the reversing ability of ACE-i(26). In the current study, improvement of relaxation was not observed. ACE-i therapy therefore seems more protective for vascular function than AT_1 receptor blocker in the 5/6 Nx model.

Another possible source of ROS might be the invasion of the arterial wall by inflammatory cells that are highly capable of ROS production. Although we cannot exclude this explanation, the focus of the current study was to explore vascular function and ROS involvement. Therefore our results suggest, that the most possible source of ROS was a vascular smooth muscle layer.

In conclusion, the present study showed that the effect of chronic treatment with the AT_1 receptor blocker losartan on CRF under experimental conditions protects the mesenteric artery from a decrease in myogenic response. Moreover, we conclude that oxidative stress acutely contributed to this phenomenon.

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Chapter 6

Vildagliptin restores renal myogenic function and attenuates renal sclerosis independently of effects on blood glucose or proteinuria in Zucker Diabetic Fatty rat

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Abstract

Introduction: Type 2 diabetes mellitus (T2DM) is associated with an increased risk for cardiovascular diseases and may lead to end organ damage, such as chronic renal failure (CRF). The ability of small arteries to constrict with increasing transmural pressure, termed myogenic constriction (MC), is part of the renal autoregulatory mechanisms. Failure of MC is associated with CRF development. The Zucker Diabetic Fatty (ZDF) rat develops type-2 diabetes, thereby representing a clinically relevant model to investigate changes in renal and peripheral vascular reactivity in settings of CRF in T2DM. Dipeptidyl peptidase-4 (DPP-4) inhibitors, a novel class of drugs to regulate blood glucose levels in T2DM, also posses a direct protective effect on the cardiovascular system. We hypothesized that renal failure in ZDF is related to the decrease in MC of intrarenal arteries and that treatment with the DPP-4 inhibitor vildagliptin prevents such changes.

Methods: Renal arteries isolated from 25 weeks old lean, ZDF and ZDF treated with vildagliptin (for 15 weeks) were transferred to an arteriograph to assess agonist and pressure induced (MC) contractile properties. Furthermore, blood glucose, proteinuria, focal glomerulosclerosis and p22phox mRNA expression of renal tissue were measured.

Results: Compared to lean controls, ZDF had significantly increased plasma glucose levels and displayed renal failure as shown by proteinuria and glomerulosclerosis. Furthermore, ZDF rats had impaired MC of renal arteries and increased renal p22phox expression. Treatment with vildagliptin did not affect plasma glucose levels or proteinuria, but effectively decreased glomerulosclerosis and restored MC and p22phox expression to levels found in lean rats.

Conclusion: Vildagliptin treatment protects diabetic rats against renal vascular changes and kidney damage part of which might be through a reduction of oxidative stress.

Introduction

Obesity is recognized as a global public health problem and during the past 6 years there has been a dramatic increase in obesity in the United States[1]. Personal and economic consequences of overweight and obesity are huge and are a high health risk in the United States and in the rest of the world[2].

The metabolic syndrome has become a major complication of obesity, characterized by parameters such as elevated plasma glucose or plasma lipid disorders[3]. The clinical consequence of the metabolic syndrome, type 2 diabetes mellitus (T2DM) is associated with an increased risk for both micro- and macrovascular complications[4;5], along with cardiovascular diseases and may lead to end organ damage, such as chronic renal failure (CRF) [6;7].

Altered blood flow autoregulatory capacity of the kidney can lead to renal injury and mainly reflects altered ability of arteries to relax or constrict in response to certain stimuli. In contrast to vasodilatation, vasoconstriction in diabetes is less widely investigated. However, vasoconstriction is a main regulatory process in smaller arteries of the kidney.

The natural ability of small arteries to constrict at increasing transmural pressure, termed myogenic constriction (MC), represents one of most important renal autoregulatory mechanisms. The exact intracellular mechanism of MC is not yet fully understood but several pathways has been proposed[8-11]. This mechanism of vascular resistance varies with the location of the vascular bed, physiologic or pathologic conditions and age. Moreover, there are different signal transduction pathways involved in the generation of MC[12-15]. Myogenic constriction of the renal arterial bed serves to protect the kidney from damage caused by increases in and large fluctuations of intraglomerular pressure. In a spontaneous model of CRF, the Fawn-Hooded Hypertensive rat (FHH), high glomerular capillary pressure is observed as a result of low afferent arteriolar resistance and moderately high systemic blood pressure[16;17].

Thus, myogenic dysfunction can result in end organ damage such as chronic renal failure in Fawn-Hooded rat, or in the stroke prone SHR (SHRsp) rat that displays the loss of MC in cerebral circulation, which may facilitate cerebral overperfusion and hemorrhage formation in SHRsp[18]. A blunting of the constriction of afferent glomerular arteriole to pressure in streptozotocin induced diabetes may account for progression of diabetic nephropathy in rat[19]. This collectively suggests importance of MC in organ protection.

The Zucker Diabetic Fatty (ZDF) rat is a substrain of the obese Zucker rat, which develops diet-induced type-2 diabetes (T2DM) and metabolic syndrome. These rats were selected for the current study because they display similar conditions as T2DM in humans:

obesity , hyperglycemia and an abnormal lipid profile[20;21], thereby creating a clinically relevant model to investigate changes in renal and peripheral vascular reactivity in settings of CRF in T2DM.

The DPP IV inhibitors are novel drugs for T2DM treatment. Dipeptidyl peptidase-4 (DPP-4) inhibitors prolong the half-life of endogenous GLP-1, thus increasing insulin and reducing glucagon secretion. Moreover, it has been shown that DPP-4 inhibitors also posses a direct protective effect on cardiovascular system[22].

Based on current knowledge concerning chronic renal failure and myogenic constriction we hypothesized that renal failure in ZDF is connected to decreased MC of intrarenal arteries and that treatment of ZDF rats with vildagliptin prevents these changes. Furthermore, we wanted to explore whether putative changes are specific for the renal vasculature or are also found in the peripheral vasculature represented by the mesenteric artery.

Materials and methods

Animals

Male ZDF (ZDF-Leprfa/Crl; n=7), and lean littermate (ZDF-Leprfa/+/Crl; n=14) rats were obtained from Charles River (Charles River Netherland B.V., Maastricht, The Netherlands) at 8 wks of age. Animals were housed under standard conditions of temperature (21-24ºC), humidity (40-60%) and 12 h light:dark cycle at the animal facilities of the University of Groningen. All animals had free access to food (Purina LabDiet Formulab 5008, Charles River Netherland B.V., Maastricht, The Netherlands) and drinking water throughout the study. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

After 2 weeks of acclimatization, ZDF rats were randomly divided into two groups (n=7 per group) at age of 10 weeks. Immediately after measurements of baseline characteristics, one group was treated with vildagliptin (vilda; Galvus®, Novartis Pharma AG, Basel, Switzerland) at the dose 3mg/kg/day in drinking water. At 25 weeks of age, before sacrification, a catheter was placed into the carotid artery under anesthesia with 2.5% isoflurane in O2 and connected to a pressure transducer (Micro-tip 3French, Millar Instruments Inc., Houston, TX, USA) and a PC equipped with an analog-digital converter and appropriate software (Millar Instruments Inc., Houston, TX, USA). After a 10-min period of stabilization, systolic and diastolic blood pressure were measured. After blood pressure measurements, blood samples were obtained from rats via tail vein and thereafter

animals were sacrificed: kidneys and mesenteric arterial bed was excised for investigation of vascular function.

Clinical chemistry

Blood samples were analyzed for whole blood glucose, Hb1Ac and total cholesterol. Whole blood glucose levels were assessed in 2.5 µL of samples by using Accu-Check Aviva + kit (Roche Diagnostics Nederland B.V., Almere, The Netherlands). Similarly, whole blood Hb1Ac% and cholesterol was assessed in 1 µL of samples by using DCA Vantage Analyzer + kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) and Accutrend + kit (Roche diagnostics Nederland B.V., Almere, The Netherlands), respectively.

Twenty-four hour urine collections for the determination of urinary total protein were performed at the end by placing the rats in metabolic cages for 24 hours. Urinary total protein excretion was determined by end-point measurement with TCA precipitation (Nephelometer analyzer II; Dade Behring, Marburg, Germany) in 24-h urine samples.

Vascular reactivity of small renal and mesenteric resistance arteries

Small renal (interlobar) and third-order branches of superior mesenteric arteries were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously [23]. Artery segments were cannulated on glass micropipettes and the vessel chamber was continuously recirculated with warmed (37°C) and oxygenated (5% CO2 in O2) Krebs solution with a pH of 7.4. An inverted light microscope attached to a video camera and video dimension analyzer was used to continuously register lumen diameter.

To exclude the role of endothelium in myogenic constriction of studied arteries, endothelium removal was performed by perfusing the artery with 5 ml of air. The removal was confirmed by the absence of dilation to acetylcholine ($3x10^{-5}$mol/l) following a submaximal pre-constriction with phenylephrine ($3x10^{-7}$mol/l).

Myogenic reactivity of small mesenteric resistance arteries

Intraluminal pressure was set at 60 mmHg, arteries were allowed to equilibrate for 45 minutes. Following a stabilization period, intraluminal pressure was decreased to 20 mmHg and myogenic reactivity was studied by obtaining active pressure-diameter curves over a pressure range of 20-160 mmHg in steps of 20 mmHg. Each pressure step was maintained for 5 minutes to reach the stable contractile response. Thereafter, calcium containing Krebs solution was exchanged for calcium-free Krebs solution supplemented with ethyleneglycol-

bis-(b-aminoethylether)tetraacetic acid (EGTA, 2 mmol/l) and passive pressure-diameter curves were obtained over the same 20-160 mmHg pressure range.

Smooth muscle reactivity of renal arteries

Prior to myogenic reactivity assessment arteries where examined for potential differences in depolarization and/or receptor mediated smooth muscle contractility. After stabilization period, dose response curves were obtained for phenylephrine (PE) and KCl with a wash out period between protocols.

Solutions and drugs

Vessel segments were perfused with Krebs solution of the following composition (in mmol/L): NaCl 120.4, KCl 5.9, CaCl2 2.5, MgCl2 1.2, NaH2PO4 1.2, glucose 11.5, NaHCO3 25.0. All other compounds were purchased from Sigma (St. Louis, MO, USA).

RNA isolation and real time PCR

Frozen kidney samples were homogenized, and RNA was isolated using a Qiagen kit (Qiagen, Venlo, The Netherlands), which included a DNAse step. Integrity of RNA was determined using agarose gel electrophoresis, and the RNA concentration was measured spectrophotometrically at 260 nm. RNA (1 µg) was reverse-transcribed, and cDNA was further used to analyze rat p22phox gene expression using a real-time PCR protocol, as described elsewhere[24]. Sequence-specific PCR primers were purchased from Biolegio (Nijmegen, The Netherlands). The sequences of the primers used were as follows: p22phox forward:5'GCTCATCTGTCTGCTGGAGTA3',reverse:5'ACGACCTCATCTGTCACTGG A-3'. All PCR tubes, PCR plates and disposables were purchased from Greiner bio-one, the Netherlands.

Data analysis and calculations

Data are expressed as mean ± SEM; n values represent the number of investigated rats as well as the number of investigated arteries since one artery segment per rat was used for the same protocol. To characterize myogenic responsiveness, the following parameters were calculated from the pressure-diameter curve of each individual artery.

Myogenic tone, describing myogenic behavior of an artery at a given pressure, was expressed as percent decrease in active diameter from the maximally dilated (passive) diameter determined at the same pressure in calcium-free/EGTA solution, i.e., myogenic constriction (%) = 100 [(DCa-free – DCa)/DCa-free], where D is the diameter in calcium-

free (DCa-free) or calcium-containing (DCa) Krebs. For every individual artery segment maximal myogenic tone was determined as the maximal value over the studied pressure range. Concentration-response curves to vasoconstrictors KCl and PE were calculated as a percentage change from baseline artery diameter. Statistical differences between studied parameters were determined by ANOVA followed by *post hoc* test for multiple comparisons. Differences were considered significant at $p<0.05$ (two-tailed).

Results

ZDF rats developed T2DM associated renal failure

Metabolic parameters (Table 1.) were dramatically different between the groups. ZDF rats developed clear signs of metabolic syndrome associated type 2 diabetes mellitus, as evidenced by increased plasma glucose level, doubling of Hb1Ac and a significant increase in cholesterol levels in ZDF compared to lean (Table 1). Moreover, ZDF rats developed renal failure as evidenced by development of proteinuria, and kidneys from ZDF rats showed a significantly higher incidence of focal glomerulosclerosis (FGS) when compared to lean rats (Table 1).

Effect of vildagliptin treatment

Surprisingly, treatment with vildagliptin of ZDF rats had no effect on plasma glucose levels, cholesterol, Hb1Ac but this treatment led to significant reduction of DPP-4 activity (Table 1) and increase of GLP-1 plasma levels (Table 1).

Vildagliptin significantly decreased oxidative status of ZDF rats as evidenced by restored levels of p22phox mRNA in renal tissue. Furthermore, treatment with vildagliptin significantly decreased incidence of focal glomerulosclerosis (FGS) but had no effect on proteinuria. There was no significant difference found in systolic and diastolic blood pressure among the ZDF, lean and treated ZDF rats (Table 1).

Table. *Metabolic parameters of 25 weeks old animals of which ZDF rats show clear signs of type 2 diabetes mellitus associated renal failure.*

	lean	**ZDF**	**ZDF + vildagliptin**
Body weight *(g)*	387±11	410±19	408±8
Plasma Glucose *(mmol/L)*	9.6±0.4	23.9±1.7*	21.5±2.3*
Hb1Ac *(%)*	3.6±0	9.5±0.2*	9.0±0.2*
Total Cholesterol *(mmol/L)*	4.0±0.1	7.3±0.2*	6.5±1.3*
Systolic Blood Pressure *(mmHg)*	117±4	124±4	120±7
Diastolic Blood Pressure *(mmHg)*	71±4	79±4	74±5
Proteinuria *(mg/24 h)*	15.1±0.8	337.3±39.8*	370.1±66.3*
FGS *(%)*	6.3±0.7	27.9±1.3*	13.7±2.3*#
DPP-IV activity *(AU)*	98.8±22.6	140.2±21.3	64.5±22.7*
GLP-1 *(pmol/l)*	5.4±0.2	6.1±0.4	32.4±7.1* #

*Data are expressed as mean ± SEM. *p<0.05 vs ZDF, # p<0.05 vs lean*

Myogenic constriction of renal artery

Renal intralobar arteries without endothelial layer from ZDF rats showed a decreased myogenic constriction when compared to lean rats. Vildagliptin treatment effectively restored MC to values found in lean rats (Figure 1A).

Pressure independent contractility of renal artery – PE, KCl

To investigate whether impaired myogenic reactivity of renal arteries is limited to myogenic behavior only or to a general impairment of vascular contractility, and whether improvement of MC of ZDF rats by vildagliptin treatment was not due overall increased contractility of vascular smooth muscle, we investigated receptor mediated and non receptor mediated depolarization to PE and KCl. Surprisingly, cumulative dose of PE elicited a higher response in renal arteries isolated from ZDF rats when compared to lean. Treatment of ZDF rats with vildagliptin significantly decreased sensitivity of ZDF renal arteries to PE (Figure 1B). Responses to the non-receptor dependent depolarizing agent KCl did not significantly differ among the groups (Figure 2).

Therefore impaired MC of renal arteries isolated from ZDF rats is endothelium independent and it is not caused due to generally lowered contractile properties of arterial smooth muscle.

Furthermore, improvement of myogenic constriction of renal arteries of ZDF treated with vildagliptin was not caused by increased contractility of smooth muscle, endothelium independent, and specific to myogenic component only.

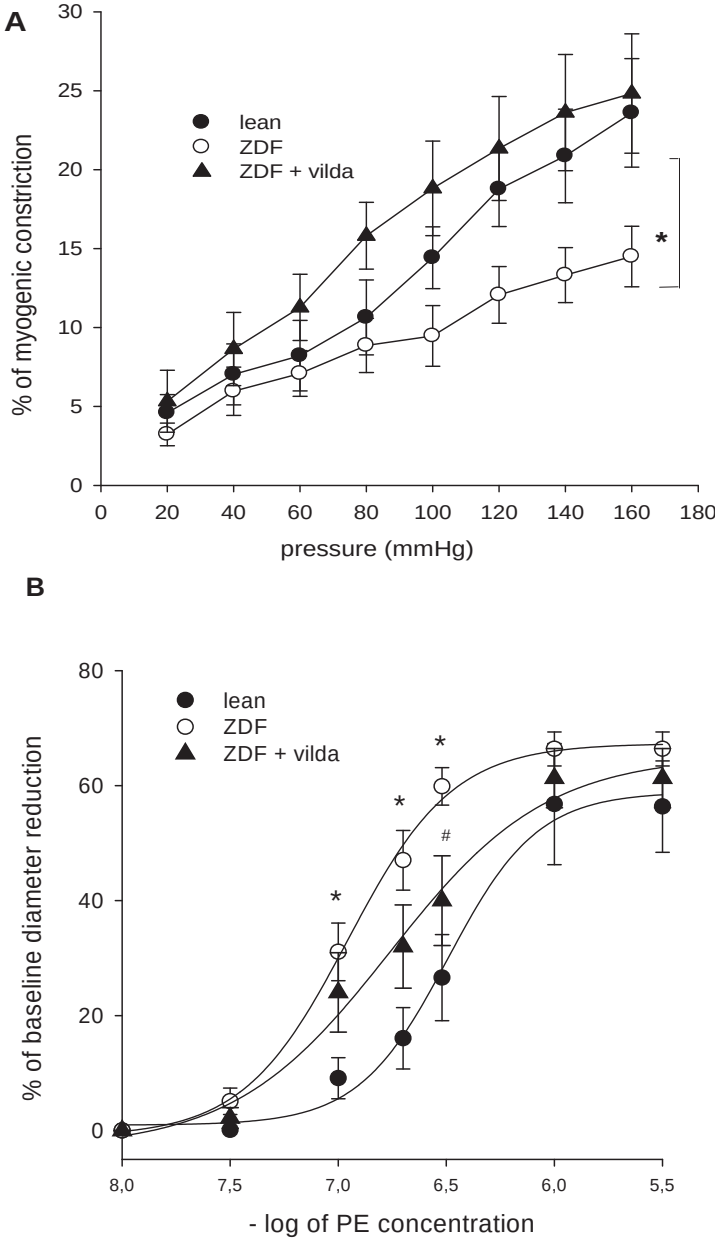

Figure 1A. *Myogenic constriction of renal arteries expressed as % of passive diameter.* ***B)*** *Dose-response curves of cumulative doses of phenylephrine (PE). Studied in renal arteries of lean, ZDF and ZDF rats treated with vildagliptin. Data are given as mean ± S.E.M. * p<0,05 compared with lean rats. # p<0, 05 compared to ZDF rats*

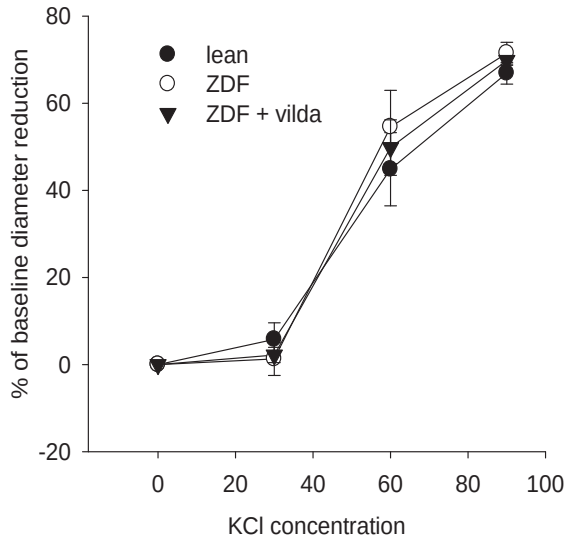

Figure 2. *Dose-response curves of renal arteries for potassium chloride (KCl Studied in lean, ZDF and ZDF rats treated with vildagliptin. Data are given as mean ± S.E.M.*

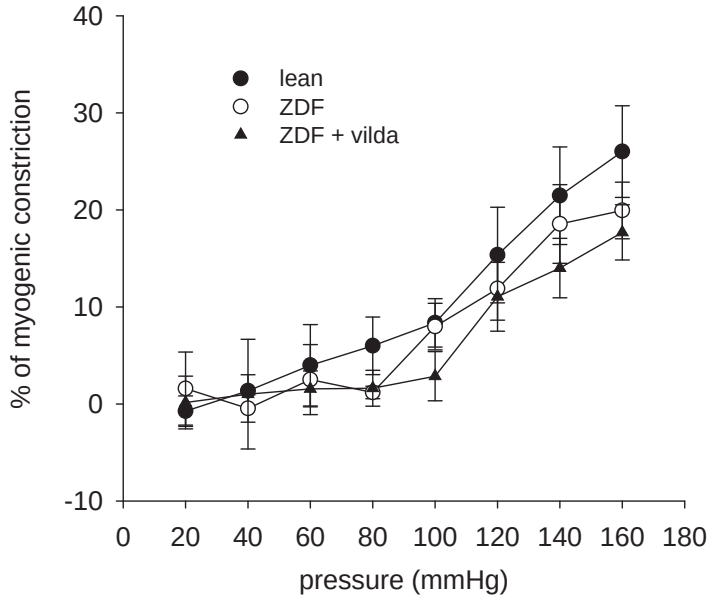

Figure 3. *myogenic tone of mesenteric arteries expressed as % of passive diameter. Data are given as mean ± S.E.M.*

Myogenic constriction of mesenteric artery

In order to explore whether differences of myogenic constriction are confined to renal artery only, or to generalized phenomenon in peripheral vasculature, MC was assessed also in mesenteric arteries. In contrast to renal arteries, myogenic constriction of mesenteric arteries did not differ between the lean, ZDF and treated ZDF rats (Figure 3).

mRNA expression of p22phox

The ZDF rats had clearly increased expression of NADPH oxidase subunit p22phox when compared to lean rats and treatment of ZDF rats with vildagliptin effectively restored mRNA levels of p22phox to lean rats (Figure 4).

Figure 4. *Treatment of ZDF rats with vildagliptin lowered mRNA p22phox expression in renal tissue. Studied in lean, ZDF and ZDF rats treated with vildagliptin. Data are given as mean ± S.E.M.*
** p<0,05 compared with lean rats. # p<0,05 compared to ZDF rats.*

Discussion

In the present study, we provide evidence that treatment of diabetic ZDF rats with DPP-4 inhibitor vildagliptin, despite having no effect on plasma glucose, cholesterol and other metabolic parameters, restored the myogenic constriction in renal arteries, and decreased glomerulosclerosis and the expression of p22phox in renal tissue. Treatment with vildagliptin had no effect on proteinuria or blood pressure. This is the first study demonstrating that vildagliptin treatment protects diabetic rats against renal vascular changes and kidney damage apart from effect on metabolic parameters. Part of the effect of vildagliptin might be through a reduction of oxidative stress.

Clearly, ZDF displayed T2DM and is a relevant model for diabetes associated renal damage. The ZDF rats had high plasma glucose and Hb1Ac levels, demonstrating impaired glucose handling. Moreover, ZDF rats developed renal damage demonstrated by increased proteinuria and glomerulosclerosis, as commonly observed in diabetes. Diabetes associated renal damage of ZDF rats is most likely a result of impaired renal autoregulation[25], as previously described in this and other models of T2DM and in clinical conditions as evidenced by glomerular hyperfiltration[26]. We show for the first time, that ZDF rats have decreased MC of renal interlobar arteries, which might account for impaired renal autoregulation. Previously, a blunted response of glomerular afferent arteriole to pressure was found in rat in which diabetes mellitus type I had been induced with streptozotocin [27]. In addition, decreased MC was also observed in small arteries isolated from gluteal fat biopsies from patients with T2DM[28], which is in line with the current results. Our observation suggests that altered myogenic reactivity may result in impairment of autoregulation of the kidney, leading to increased glomerular pressure, hyperfiltration and subsequent renal injury. This might explain the observed renal impairment in ZDF rats, although the cause and mechanism of decreased MC in this model remains unclear.

Diabetes is, however, known for elevated production of ROS, which are thought causative in the development of endothelial dysfunction. Nevertheless, some results suggest that ROS also cause a decrease in MC as well, via formation of peroxinitrite by superoxides produced by vascular smooth muscle and NO released from the endothelium[29]. In accord, *Chander et al* showed blunting of renal damage in ZDF treated with peroxinitrite scavenger[30]. We found increased expression of p22phox in renal tissue of ZDF rats, which is in accord with observations, is other diabetic models and the general concept of damaging role of ROS in

diabetes. As p22phox is a subunit of NOX, in kidney predominantly of NOX-2 localized in smooth muscle of renal vessels[31], we suggest that impaired MC in caused by increased production of NOX-2 derived ROS. Taken together, overproduction of smooth muscle ROS impairing MC and disturbing autoregulation represents an obvious mechanism of renal damage in ZDF rats.

Other possible causes of impaired and improved MC

It is generally believed, that high blood pressure influences MC. We did not observe any significant difference in blood pressure between groups, therefore excluding blood pressure as a determinant of both decreased MC in ZDF, and improvement of MC in vildagliptin treated ZDF rats. One of the other possible reasons of decreased myogenic reactivity might be the generally lowered ability of the arterial wall to constrict. However, interlobar arteries show a similar response to the nonspecific contractile agent KCl in all groups, suggesting that the mechanism(s) underlying the decrease of MC in ZDF and its restoration by vildagliptin are too some extent specific to the generation of myogenic constriction. Thirdly, disturbance of endothelial function may affect MC. *Ito et al*[32] observed endothelium mediated decrease of MC in the ophthalmic artery from BBZDR/Wor rats, a T2DM model, after exposure to high glucose, suggesting that hyperglycemia may be the reason of decreased MC. However, our current study seems to rule out direct involvement of endothelium due the fact that experiments were performed on endothelium freed arterial segments, suggesting a direct involvement of arterial smooth muscle in myogenic dysfunction.

Treatment of ZDF rats with vildagliptin did not lead to the desired effect of lowering plasma glucose. The lack of effect of vildagliptin seems not to be caused by inadequate dosing, as treated rats showed both suppressed DPP-IV activity and increased GLP-1 levels compared to untreated ZDF. Also, *Burkey et al.* reported 3 mg/kg vildagliptin to be effective in lowering glucose in insulin-resistant rat[33]. Thus, we cannot fully explain why vildagliptin did not successfully improve the metabolic condition. Possibly, ZDF rat has a genetic background precluding its effectiveness.

Despite the lack of effect on the diabetic state, vildagliptin improved MC and normalized expression of p22phox. Moreover, vildagliptin treatment strongly decreased glomerulosclerosis, but did not affect proteinuria. We assume that normalization of the increased expression of renal p22phox in ZDF in vildagliptin treated animals was most likely due to the effect of GLP-1. The GLP-1 receptor has been detected in vascular smooth

muscle and renal vasculature in human, rat and mouse[34;35]. Stimulation of this receptor may have led to improved MC, limiting the increase in glomerular pressure and glomerulosclerosis. Moreover, vildagliptin not only improved MC, but also normalized the increased responsiveness of renal arteries from ZDF to phenylephrine (PE). This suggests that vildagliptin is generally protective on vascular smooth muscle in diabetic conditions, perhaps by limiting oxidative stress. Indeed, GLP-1 receptor agonists have the potential to limit the impact of oxidative stress. Exendin-4, a GLP-1 analogue, is capable to induce the oxidative defense genes such as HO-1 and NQO1, and prevent senescence in human endothelial cells when exposed to oxidative stress[36]. Further, exendin-4 protects β-cell from apoptosis by blocking JNK and GSK3 induced by oxidative stress[37], and decreases ROS production in islet cells from Goto-Kakizaki rat via Epac mediated suppression of Src[38]. GLP-1 receptor agonists have also been reported to exert beneficial vascular effects via independently of limitation of ROS production. Exendin-4 reduced intimal thickening after vascular injury and in vitro exendin-4 was shown to attenuate proliferation of vascular smooth muscle cells (VSMCs) upon PDGF stimulation, independently of the canonical GLP-1 signaling, perhaps via exendin-4 mediated uncanonical activation of AMPK [39]. Moreover, the effect of GLP-1 appears to have direct impact on modulation of vascular function. Vasodilative properties of GLP-1 and its metabolites on rat mesenteric arteries, pulmonary circulation or aorta were described[40-42], which might partially account for cardioprotective effect in ischemia reperfusion injury models. Also, GLP-1 was found to lower blood pressure thus limiting renal and cardiac damage caused by hypertension in Dahl salt sensitive rats[43]. In addition, DPP-IV inhibitors and GLP-1 receptor agonists protect hearts from ischemia reperfusion injury[44;45]. Thus, it seems conceivable that the protective effect of vildagliptin on MC might is due to a direct effect of GLP-1 on VSMC.

Finally, renal protection of vildagliptin was limited to the decrease of glomerulosclerosis but did not include improvement of proteinuria. When searching for possible explanation several assumptions come into the consideration; One of the reasons might be based on recent findings that high glucose causes dysfunction of glomerular glycocalyx[46], as a protein-restrictive layer, which in high glucose condition is permeable for albumin, thus dissociating between diabetic and non-diabetic proteinuria. In keeping with this, as treatment did not affect glucose levels, vildagliptin treatment did not restore proteinuria, while slowing down the progression of glomerulosclerosis. The other reason of vildagliptin limited renal protection might be, that podocytes which play crucial role in development of proteinuria in renal disease do not express GLP-1 receptor. We assume that beneficial

effect of vildagliptin was mediated via GLP-1 receptor in vascular smooth muscle of renal vessels. Up to our best knowledge so far there are no reports on the presence of GLP-1 receptor in podocytes. This may explain why the renal protection of vildagliptin was limited to improvement of glomerulosclerosis and had no effect on proteinuria. The precise mechanism of action of vildagliptin to reduce FGS and maintain MC remains to be established.

Conclusion

Vildagliptin treatment restored loss of MC of renal vasculature in diabetic ZDF and prevented kidneys from developing glomerulosclerosis without improving the diabetic state. Thus, here we show for the first time and extend the knowledge on blood glucose independent effects of vildagliptin to kidney in T2DM. This collectively suggests that DPP-IV inhibitors to exert additional beneficial effects in T2DM.

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Chapter 7

Epidermal growth factor receptor inhibitor PKI-166 governs cardiovascular protection without beneficial effects on the kidney in hypertensive 5/6 nephrectomized rats

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(submitted)

ABSTRACT

Epidermal growth factor receptor (EGFR) activation is implicated in chronic kidney disease (CKD) and hypertension, as angiotensin II type 1 receptor and α1-adrenoceptor transactivate the EGFR. As the therapeutic potential of EGFR inhibition in these conditions is currently unknown, we studied the effect of the EGFR kinase inhibitor PKI-166 on CKD and associated hypertension in the rat remnant kidney model by assessing its effects on kidney, heart and vessels. CKD was induced by 5/6 nephrectomy and rats were treated with the EGFR kinase inhibitor PKI-166 (50 mg/kg/day), lisinopril (5 mg/kg/day) or vehicle from week 6 after disease induction until week 12. Nephrectomized rats displayed characteristic features of CKD including severe proteinuria, hypertension, decreased creatinine clearance, increased glomerular sclerosis, renal enlargement and left ventricular hypertrophy. Despite complete absence of effects on renal damage, PKI-166 treatment attenuated the progression of hypertension and normalized cardiac function to a similar extent as lisinopril. Moreover, PKI-166 treatment fully restored the impaired contraction of thoracic aortic rings to phenylephrine and angiotensin II and normalized the myogenic tone of mesenteric artery, as did lisinopril. Thus, blockade of the EGFR pathway exerts cardiovascular benefits in CKD without limiting the progression of renal injury. Our findings provide evidence for EGFR signaling as a target in CKD associated cardiovascular complications.

INTRODUCTION

The major cause of death in chronic kidney disease (CKD) is associated cardiovascular (CV) complications[1] Hypertension represents the most common CV complication in patients with CKD; it not only predicts mortality but also represents one of the major determinants of progression of renal injury[2] The mechanism of development of hypertension in CKD is complex and several hypotheses have been put forward including activation of renin–angiotensin (RAS)[3] and sympathetic nervous systems[4] Independently of the origin of hypertension in CKD, the increase in blood pressure leads to a progression of renal injury, thereby initiating a vicious circle[5]. Breaking this vicious cycle may provide a superior CV outcome in renal disease.

In recent years, a strong connection between hypertension and epidermal growth factor receptor (EGFR) signaling has been demonstrated. First, in genetic[6] and experimental[7] models of hypertension, an enhanced expression level of EGFR and a significant correlation between maximal epidermal growth factor (EGF) binding capacity in the aorta and blood pressure was shown. Subsequent reports in a model of spontaneous hypertension (SHR rat) substantiated these findings by demonstrating EGF to act as a potent vasoconstrictor of arteries[8] and by increased EGFR expression levels in hypertrophied left ventricle (LV).[9] Also, chronic inhibition of EGFR by the kinase inhibitor AG1478 and EGFR antisense oligonucleotides attenuate the vasoconstriction and the elevation of blood pressure in angiotensin II (Ang II)-induced hypertension.[10-11] Because metalloproteinase (MMP) inhibitors successfully prevented cardiac remodeling induced by Ang II, G protein coupled receptor (GPCR)-induced shedding of heparin binding EGF-like growth factor (HB-EGF) and subsequent EGFR transactivation is likely to play an important role in this model of hypertension.[12]

A growing body of evidence demonstrates various GPCRs to be implicated in hypertension and associated oxidative stress, cardiac hypertrophy and vascular remodeling.[12-14] Recently, it has been recognized that the transactivation of EGFR by many different $G_{q/11}$ protein-coupled receptors may constitute an important part of EGFR signaling.[15] Therefore, instead of targeting the various GPCRs, e.g. Ang II type 1 (AT_1) receptor or α_1-adrenoceptor (α_1-AR), it is conceivable that blockade of EGFR transactivation may have a significant potential in several CV conditions including hypertension,[15] heart failure,[16] cardiac[10, 17] and vascular hypertrophy.[18] In support of this, we recently showed that transactivation of the EGFR is part of the α_1-AR induced contraction of rat aorta.[19] Furthermore, transactivation of the EGFR may also play a role in renal disease[20] and/or associated CV complications.

Several experimental studies suggest EGFR inhibitors to possess renoprotective effects[21-23]. These findings collectively imply the possible therapeutic potential of EGFR inhibition in hypertension, as hypothesized recently.[15,24] However, to what extent EGFR signaling is involved in the progression of CKD and/or associated hypertension is yet unknown. To investigate the therapeutic potential of EGFR inhibitors in renal disease and associated CV complications, the effects of PKI-166 were investigated in 5/6 nephrectomized (5/6Nx) rats, by assessment of renal function and damage, blood pressure and cardiac parameters. In addition, we assessed vasoreactivity in isolated vessels by measuring sensitivity to AT_1 receptor and α_1-AR stimulation (aorta) and myogenic tone (mesenteric artery). Rats treated with an angiotensin converting enzyme (ACE) inhibitor (lisinopril) and vehicle served as positive and negative control groups.

METHODS

Animals

Experiments were performed on 12 weeks old male Wistar rats (n=60, 330 to 400 g, Harlan, Zeist, the Netherlands). Animals were housed under standard conditions of temperature (21-24ºC), humidity (40-60%) and 12 h light:dark cycle at the animal facilities of the University of Groningen. Animals had free access to food (standard rat chow; Hope Farms, Woerden, the Netherlands) and drinking water throughout the study. Animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the Groningen University Medical Center.

Experimental Protocol

After obtaining blood samples and baseline measurements of SBP and DBP, rats underwent right nephrectomy and resection of two-thirds of the left kidney by ligation of 2-3 branches of the left renal artery.[25] Sham operated rats underwent the same procedure without the surgical reduction of kidney mass.

Six weeks after the operation, 5/6Nx rats were treated with either vehicle (5/6Nx+Vehicle; *n*=12) or EGFR kinase inhibitor (5/6Nx+PKI-166; *n*=10) or lisinopril (5/6Nx+Lisinopril; *n*=8) until the week 12. Sham animals received either vehicle (Sham+Vehicle; *n*=10) or EGFR kinase inhibitor (Sham+PKI-166; *n*=12). PKI-166 (50 mg/kg/day), lisinopril (5 mg/kg/day) or vehicle treatment was provided daily by gavage. PKI-166 was dissolved in

10% DMSO+0.5% Tween-80 diluted 1:20 (vol/vol) in water. During the treatment period, proteinuria and tail-cuff blood pressure was assessed every 3 weeks.

At the end of the protocol, under short anesthesia with 2.5% isoflurane in O_2, cardiac performance was measured by a pressure transducer catheter which was inserted through the right carotid artery (Micro-Tip 3-French; Millar Instruments Inc., Houston, Tex., USA). Heart rate, LVEDP, LVSP, and the maximal rates of increase and decrease in LV pressure ($+dP/dt_{max}$ and $-dP/dt_{max}$)[46] were recorded. Central systolic and diastolic blood pressures were measured after withdrawal of the catheter into the aortic root. Blood samples (2-3 mL) were collected from the abdominal aorta for biochemical analyses and heart and kidneys were harvested for further analysis. Thoracic aorta and third-order branches of superior mesenteric arteries were obtained and placed into ice-cold Krebs solution.

Proteinuria and blood pressure measurement

Rats were placed in metabolic cages for 24 h and proteinuria was determined by trichloroacetic acid precipitation (Nephelometer Analyzer II; Dade Behring, Marburg, Germany).

Blood pressure was measured by means of the tail-cuff method (PS-200A; Riken-Kaihatsu; Tokyo, Japan and IITC Life Sciences, Woodland Hills, CA, USA).[25] In brief, animals were adapted to the procedure in a 2 weeks training period before the experimental protocol. For each animal, blood pressure values represent the mean of three to five recordings obtained in a single session.

Biochemical analysis

Plasma and urine creatinine were measured by means of a photometric assay with the Jaffé method without deproteinization (DiaSys Diagnostic Systems, Holzheim, Germany) and creatinine clearance was calculated as (Urine Creatinine x Urine flow) / (Plasma Creatinine x Body Weight).

Immunohistochemistry and morphometry

Immunostainings for α-SMA and macrophage (ED-1) were performed on cryosections of the kidneys using anti-α-SMA (Clone 1A4; Sigma, St. Louis, MO, USA) and anti-ED1 (Serotec Ltd, UK) antibodies incubated for 1 h respectively at 1:15000 and 1:750 dilution, followed by horseradish peroxidase (HRP)-conjugated rabbit-anti-mouse and subsequently HRP-conjugated goat-anti-rabbit antibodies. Positive cortical interstitial and glomerular α-SMA and ED1 staining were measured by Aperio ImageScope software (version

9.1.772.1570, Aperio Technologies Inc, Vista, CA, USA) at 200x magnification. Data from α-SMA and ED1 immunostainings are presented as the intensity and the number of the positive pixels, respectively.
Renal damage was assessed in paraffin embedded sections of kidneys stained with Periodic Acid Schiff reagent. The incidence of FGS was microscopically evaluated semi-quantitatively by scoring of 50 glomeruli per slide on a scale of 0 to 4 by an examiner blinded for the groups as described previously.[47]

Vascular reactivity of mesenteric arteries

Third-order branches of superior mesenteric arteries were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously.[48]
Intraluminal pressure was set at 80 mmHg and arteries were allowed to equilibrate for 40 minutes. Subsequently, smooth muscle and endothelium viability was checked by a single dose of PE (0.3 μM) and acetylcholine (ACh; 30 μM). Following wash out, intraluminal pressure was decreased to 20 mmHg and myogenic reactivity was studied by obtaining active pressure-diameter curves over a pressure range of 20-160 mmHg in steps of 20 mmHg. Each step was held for 5 minutes to reach stable contraction. Thereafter, Krebs solution was exchanged for calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether) tetra-acetic acid (EGTA, 2 mM) and passive pressure-diameter curves were obtained over the 20-160 mmHg pressure range.

Contractility of thoracic aorta segments

Aorta segments (approximately 2 mm) were cleaned from perivascular tissue and mounted in an organ bath with Krebs solution at 37 °C and continuously bubbled with 95% O_2 and 5% CO_2. Viability of smooth muscle cells was checked by pre-constriction with PE (1 μM). After wash out and 30 minutes of stabilization, contractility was measured in response to cumulative concentrations of Ang II (1 nM –1 μM) in endothelium-denuded rings. Finally, potassium chloride (KCl; 60 mM) was added to the organ baths. In additional endothelium-denuded rings, PE (1 nM –10 μM) mediated aorta contractility was also studied. Each experimental condition was studied in duplicate rings.

Solutions and drugs

The composition of Krebs solution was (in mM): NaCl (120.4), KCl (5.9), $CaCl_2$ (2.5), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), glucose (11.5), $NaHCO_3$ (25.0) at pH 7.4. All compounds for

Krebs solution and all other drugs were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). PKI-166 was kindly provided by Dr. Giorgio Caravatti (Novartis Pharma AG, Basel, Switzerland).

Statistical analysis

Data are expressed as mean ± SEM; *n* values represent the number of investigated rats. SPSS 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. Concentration-response and myogenic constriction curves were compared by ANOVA for repeated measures followed by Bonferroni *post hoc* test for multiple comparisons. Myogenic tone was expressed as percent decrease in active diameter from the maximally dilated (passive) diameter determined at the same pressure in calcium-free/EGTA solution, i.e., myogenic tone (%)= 100 $[(D_{Ca\text{-}free} - D_{Ca})/D_{Ca\text{-}free}]$, where D is the diameter in calcium-free ($D_{Ca\text{-}free}$) or calcium-containing (D_{Ca}) Krebs. Group comparison of animal parameters was performed by One-Way ANOVA followed by Dunnett's or Bonferroni *post hoc* tests. Differences were considered significant at $P<0.05$ (two-tailed).

RESULTS

Animal characteristics and effects of 5/6Nx

Animal characteristics are presented in Table 1. Vehicle-treated 5/6Nx animals had increased kidney weight/body weight ratio, lower creatinine clearance (Table 1), and increased water intake and urinary output between weeks 5 and 11 (data not shown) compared to Sham. Body weights were similar in vehicle-treated Sham and 5/6Nx rats (data not shown). 5/6Nx rats displayed a gradual increase in proteinuria in the first 5 weeks (Figure 1, $P<0.05$ vs. Sham groups), which continued to increase in vehicle-treated rats.

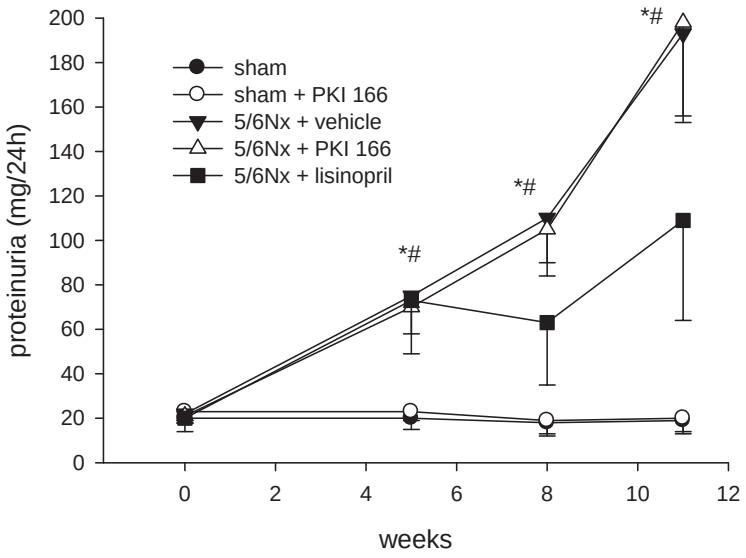

Figure 1. *The effect of treatment with PKI-166 or lisinopril on proteinuria levels. Treatments were initiated 6 weeks after induction of 5/6Nx. Data are expressed as mean±SEM. *P<0.05 versus Sham+Vehicle and #P<0.05 versus Sham+PKI-166.*

At the end of the experimental protocol, 5/6Nx+Vehicle rats had higher focal glomerulosclerosis (FGS) score (Figure 2C1 and F) and increased interstitial α-smooth muscle actin (α-SMA) staining (Figure 2C2 and G) compared to Sham (Figure 2A1 and A2). 5/6Nx+Vehicle rats showed a slight, but non-significant increase in interstitial macrophages (ED-1 immunostaining; Sham+Vehicle=0.012±0.003, Sham+PKI-166=0.007±0.002, 5/6Nx+Vehicle=0.016±0.004, 5/6Nx+PKI-166=0.014±0.003, and 5/6Nx+Lisinopril=0.017±0.009).

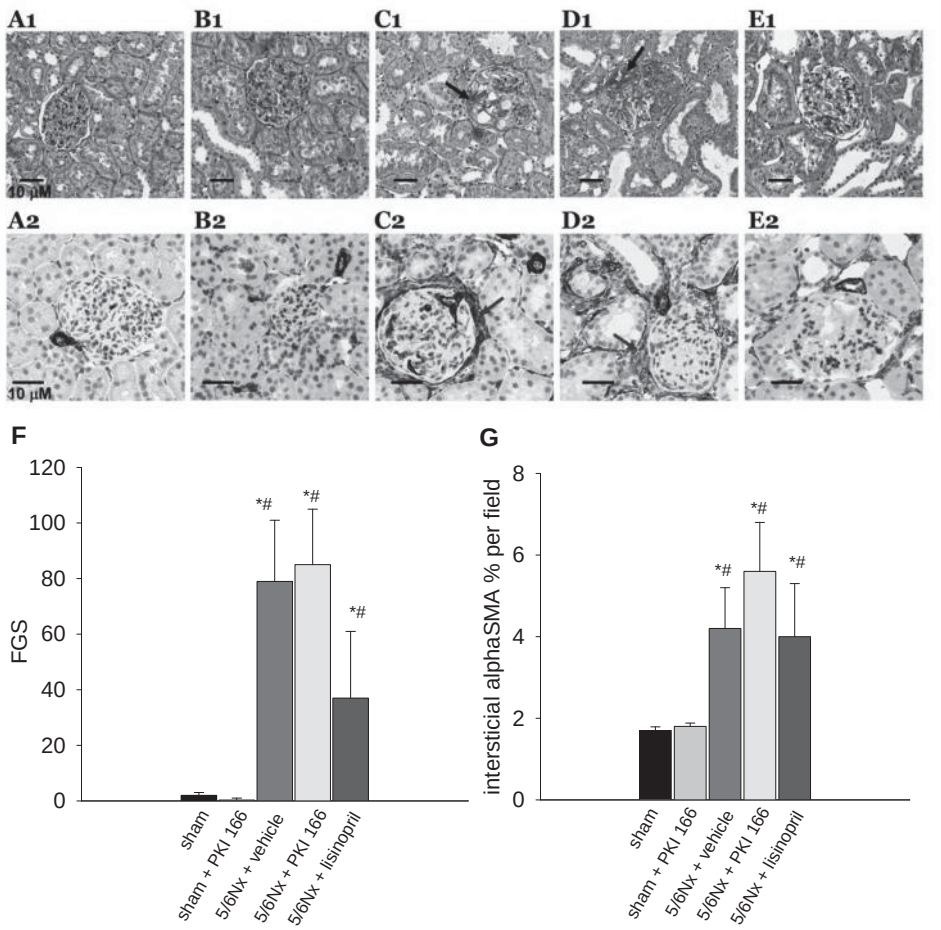

Figure 2. *Assessment of renal damage. Fifty glomeruli per slide (**A1-E1**) were microscopically evaluated and scored semi-quantitatively (**F**) for the incidence of focal glomerulosclerosis (FGS). Representative photomicrographs of kidney sections from Sham+Vehicle (**A1**), Sham+PKI-166 (**B1**), 5/6Nx+Vehicle (**C1**), 5/6Nx+PKI-166 (**D1**) and 5/6Nx+Lisinopril (**E1**) groups. For the assessment of prefibrotic changes, the intensity of the positive cortical interstitial and glomerular (**A2-E2**) pixels was measured by α-smooth muscle actin (α-SMA) staining (**G**). Representative photomicrographs of kidney sections from Sham+Vehicle (**A2**), Sham+PKI-166 (**B2**), 5/6Nx+Vehicle (**C2**), 5/6Nx+PKI-166 (**D2**) and 5/6Nx+Lisinopril (**E2**) groups. Data are expressed as mean±SEM. *P<0.05 versus Sham+Vehicle and #P<0.05 versus Sham+PKI-166.*

5/6 nephrectomy induced a significant increase in arterial systolic blood pressure (SBP) at week 5 after the operation. In vehicle-treated 5/6Nx rats, SBP continued to increase up to 8 weeks and remained stable afterwards (Figure 3A). Diastolic blood pressures (DBP) was

slightly (but non-significantly) higher in 5/6Nx animals at week 5, but increased significantly in vehicle-treated 5/6Nx rats afterwards (Figure 3B). Also, LV weight/body weight ratio, LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) were increased in vehicle-treated 5/6Nx rats compared to Sham (Table 1). Together, these data demonstrate the successful induction of experimental CKD and related changes in CV parameters induced by 5/6 nephrectomy.

The above measurements were also obtained in Sham animals treated from week 6 to 12 with PKI-166. Importantly, no difference in any of the parameters was observed in Sham+PKI animals compared to vehicle treated Sham rats (Figures 1-3, Table 1).

Table 1. *In vivo characteristics of untreated and treated sham and 5/6 Nx rats 12 weeeks after sham or 5/6 Nx operation.*

	Sham+ Vehicle	**Sham+ PKI-166**	**5/6 Nx+Vehicle**	**5/6 Nx+PKI-166**	**5/6 Nx+ Lisinopril**
Kidney weight/BW	0.35±0.02	0.33±0.01	0.49±0.02*#	0.48±0.04*#	0.43±0.01#
Creatinine clearance (mL/min/kg)	7.8±0.7	7.3±0.4	3.6±0.4*#	2.9±0.5*#	4.7±0.6*#
Heart rate (beats/min)	355±12	333±12	355±16	322±15	358±16
Left ventricular weight/BW	0.21±0.01	0.20±0.003	0.25±0.01*#‡	0.26±0.01*#‡	0.20±0.01
+dP/dt$_{max}$	9569±505	8687±462	10326±404	8900±482	8393±685
-dP/dt$_{max}$	-7252±354	-7322±314	-8762±398*‡	-7674±602	-6334±437
LVSP (mmHg)	119±4	116±4	151±5*#‡	135±7‡	108±5
LVEDP (mmHg)	4±1	3±1	13±4#†	3±1	4±1

*Data are given as means±S.E.M. LVSP: Left ventricular systolic pressure, LVEDP: Left ventricular end-diastolic pressure. *P<0.05 versus Sham+Vehicle, #P<0.05 versus Sham+PKI-166, †P<0.05 versus 5/6 Nx+PKI-166l, ‡P<0.05 versus 5/6 Nx+Lisinopril*

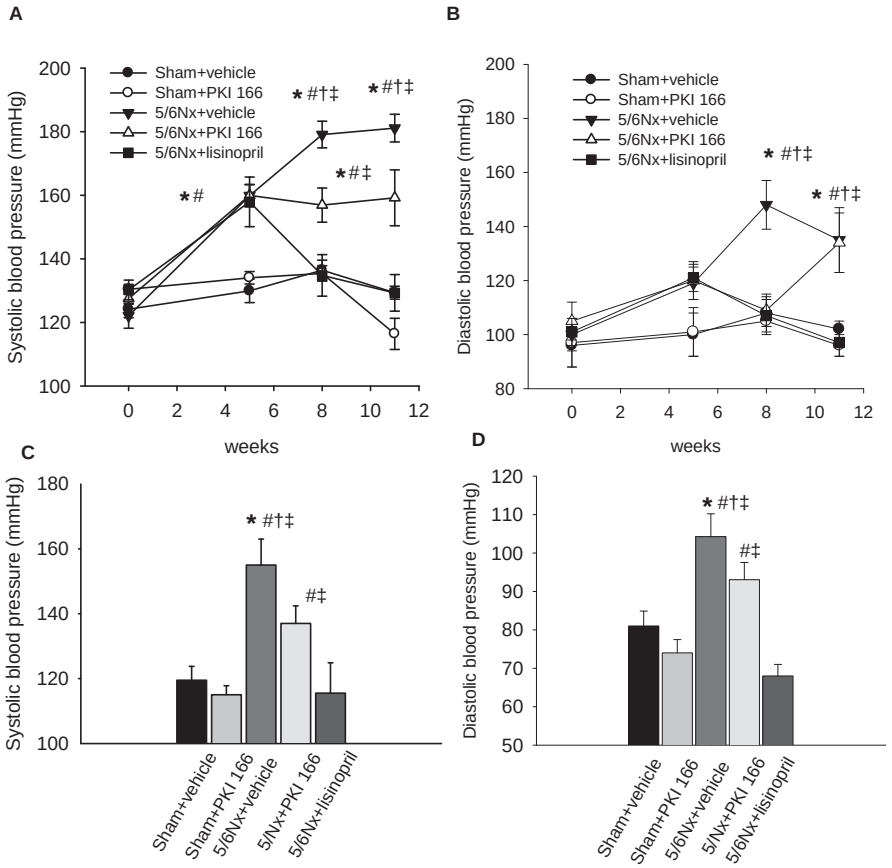

Figure 3. *The effect of treatment with PKI-166 or lisinopril on conscious arterial systolic (**A**) and diastolic blood pressure (**B**). Twelve weeks after 5/6Nx, arterial blood pressure was also measured under short anesthesia by Millar catheter (**C** and **D**). Data are expressed as mean±SEM. *P<0.05 versus Sham+Vehicle, #P<0.05 versus Sham+PKI-166, †P<0.05 versus 5/6Nx+PKI-166, and ‡P<0.05 versus 5/6Nx+Lisinopril.*

The effects of PKI-166 on renal damage

Six weeks of treatment with PKI-166 between weeks 6 and 12 after 5/6 nephrectomy did not prevent increased water intake and urinary output (data not shown). Neither PKI-166 nor lisinopril prevented renal hypertrophy (Table 1). Creatinine clearance was decreased in PKI-166-treated 5/6Nx rats, similarly to vehicle-treated rats, and to a lesser extent in lisinopril-treated 5/6Nx rats (Table 1). Whereas PKI-166 did not affect the progression of proteinuria throughout the treatment period, lisinopril treatment prevented the increase in

proteinuria (Figure 1). In line with proteinuria data, PKI-166 treatment did not affect both FGS score (Figure 2D1 and F) and interstitial α-SMA (Figure 2D2 and G) staining. In contrast, lisinopril treatment partially protected the kidneys from injury as evidenced by a lower FGS score (Figure 2E1 and F). Collectively, these data demonstrate that PKI-166 treatment did not affect the parameters of kidney injury after 5/6 nephrectomy, whereas lisinopril limited the progression of renal disease.

The effects of PKI-166 on hypertension and cardiac function

Treatment with PKI-166 blunted the increase in SBP in 5/6Nx animals and completely restored DBP to Sham levels, whereas lisinopril completely restored both (Figure 3A and B). At sacrifice (i.e. 12 weeks after the induction of 5/6 nephrectomy), SBP and DBP was also measured under short anesthesia by Millar catheter. In accord with the conscious arterial blood pressure measurements, PKI-166 treatment significantly lowered SBP and DBP (Figure 3C and D). Lisinopril completely restored the increased SBP and DBP to Sham levels at week 12 (Figure 3C and D).

Neither PKI-166 nor lisinopril significantly influenced the heart rate (Table 1). On the other hand, PKI-166 did not prevent the increase in LV weight, while lisinopril treatment did (Table 1). Nevertheless, the increase in LVSP of vehicle-treated 5/6Nx rats was attenuated by PKI-166 and lisinopril restored the LVSP to Sham values (Table 1). Remarkably, PKI-166 completely prevented the increase in LVEDP after 5/6Nx, as lisinopril did (Table 1).

Vascular effects of 5/6Nx and PKI-166

To further explore the alterations in systemic vascular reactivity after 5/6 nephrectomy and the effects of PKI-166 on altered responses, we investigated myogenic constriction in the mesenteric artery and sensitivity to GPCR agonists in the thoracic aorta.

Myogenic constriction

Passive diameters of mesenteric arteries did not differ among the experimental groups over the pressure range (Figure 4A), suggesting no apparent changes in maximal relaxant ability of the investigated arteries. Active diameters were increased only in the 5/6Nx+Vehicle group (Figure 4B), signifying a gross impairment of myogenic constriction in mesenteric artery at 12 weeks after 5/6Nx (Figure 4C), as reported previously[25]. Chronic treatment of 5/6Nx rats either with PKI-166 or lisinopril completely restored the impaired myogenic tone to the Sham values (Figure 4C).

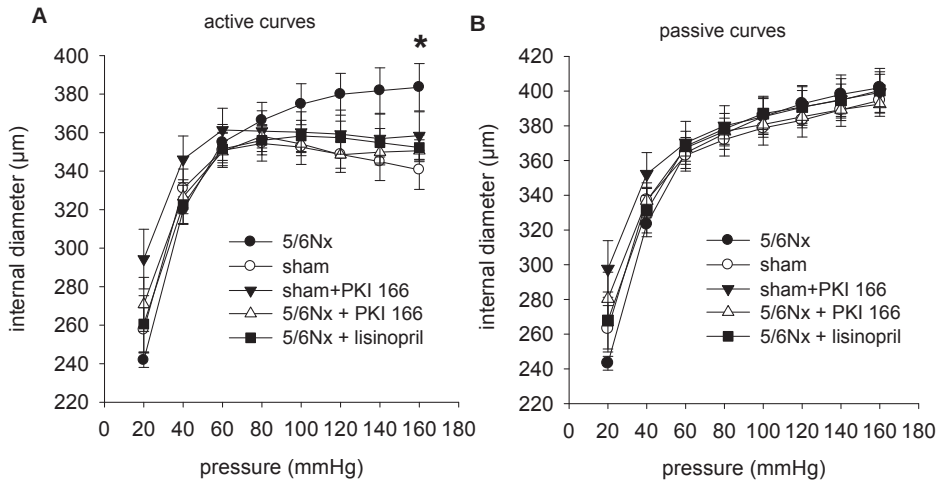

Figure 4. *Vascular reactivity of small mesenteric arteries. Diameters of small mesenteric arteries in response to stepwise increase of intraluminal pressure in the absence (**A**) or presence of calcium (**B**) Data are expressed as mean±SEM. *P<0.05 versus all groups*

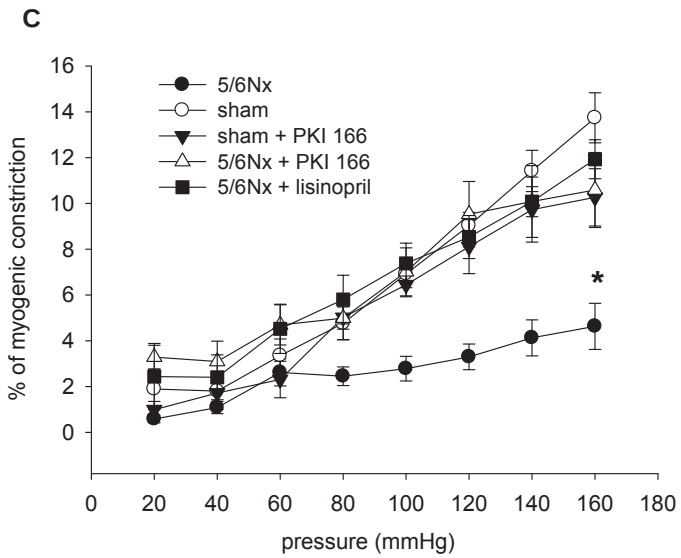

Figure 4C*) Vascular reactivity of small mesenteric arteries. Myogenic tone expressed as % of passive diameter. (Data are expressed as mean±SEM. *P<0.05 versus all groups*

Ang II and phenylephrine mediated aorta contractility:

To assess the involvement of EGFR in Ang II and phenylephrine (PE) mediated contraction in thoracic aorta rings, full concentration-response curves of Ang II and PE were obtained in 5 to 6 rats per experimental group. Twelve weeks after 5/6 nephrectomy, contraction response to Ang II was significantly diminished in thoracic aorta (Figure 5A). The contractile response to Ang II in 5/6 nephrectomy was partially restored by PKI-166 and completely by lisinopril (Figure 5A). Similarly to our findings with Ang II, PE mediated aorta contractility was attenuated in 5/6Nx+Vehicle group (Figure 5C). Both lisinopril and PKI-166 completely restored the impaired PE mediated contractions (Figure 5C).

To investigate the role of hypertension in the attenuated Ang II and PE mediated aorta contractility, we analyzed the relationship between arterial SBP and maximal contractile response of aortic rings. A negative correlation between SBP and the maximal contraction response to Ang II (Figure 5B; R=-0.515, P<0.01) and PE (Figure 5D; R=-0.848, P<0.0001) was found.

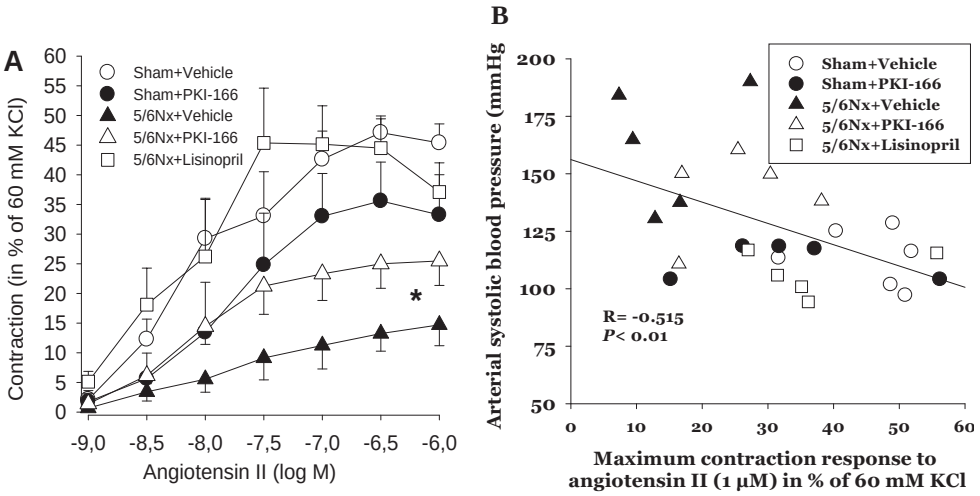

Figure 5. *Full concentration-response curves of angiotensin II (**A**) mediated contraction and relationship between arterial SBP and maximal contraction response of aortic rings to angiotensin II (**B**) in rat thoracic aorta rings. Data are expressed as mean±SEM. *P<0.05 versus all groups.*

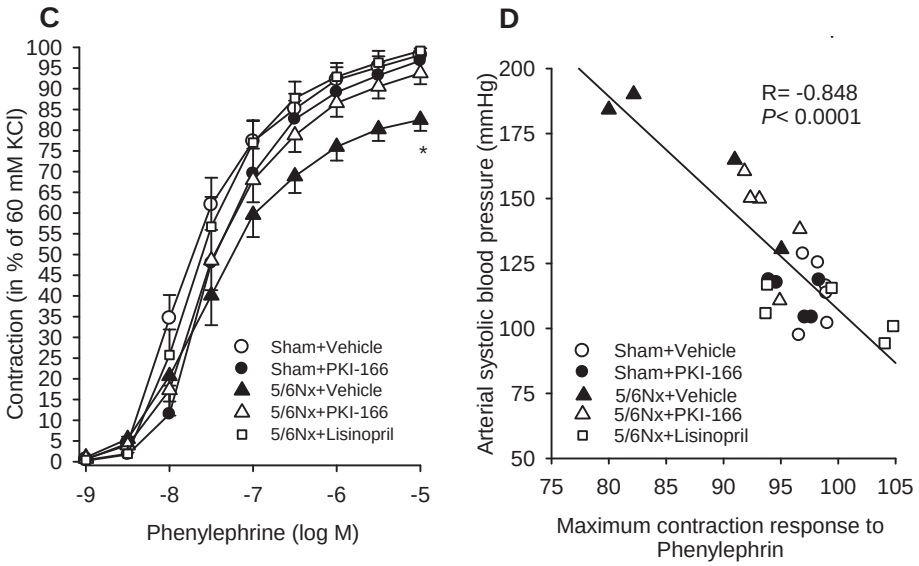

Figure 5. *Full concentration-response curves of phenylephrine (**C**) mediated contraction and relationship between arterial SBP and maximal contraction response of aortic rings to phenylephrine (**D**) in rat thoracic aorta rings. Data are expressed as mean±SEM. *P<0.05 versus all groups.*

DISCUSSION

Our findings show that chronic inhibition of the EGFR by PKI-166 prevents the progression of hypertension and limits cardiovascular changes, independent of limiting the progression of functional and structural changes in the kidney in 5/6 nephrectomy in rat. The beneficial effect of PKI-166 treatment on the CV system is substantiated by preservation of LVEDP and by the normalization of the impaired myogenic tone and contractile response of isolated arteries. These data collectively indicate that CV protective effects via EGFR inhibition in kidney disease are independent of modulation of renal injury.

In this study, 5/6Nx rats displayed the characteristic features of CKD, as evidenced by severe proteinuria, decreased creatinine clearance, increased glomerular sclerosis, and renal hypertrophy. In line with our previous findings, CKD was accompanied by an increase in

blood pressure and a gross reduction in the development of myogenic tone in small mesenteric arteries. The reduced myogenic tone might represent a compensatory mechanism counteracting the increase in peripheral resistance in CKD.[25] Furthermore, reduction of myogenic tone was completely restored by both treatments, suggesting a link between myogenic tone and blood pressure. We previously demonstrated that ACE inhibition normalizes myogenic tone of mesenteric arteries, while attenuating elevated blood pressure and the progression of renal injury.[25] In that study, we were unable to dissociate whether the effect of ACE inhibition on myogenic tone is due to lowering blood pressure or mitigation of the renal impairment. This study extends our previous findings as EGFR inhibition reduced blood pressure and fully restored myogenic tone, while not redressing any renal parameters. Thus, most likely, the reversal effect of ACE inhibitors on peripheral myogenic tone in kidney disease is due to their antihypertensive effect.

Renal disease related cardiac-specific alterations were also observed in vehicle-treated 5/6Nx rats, including LV hypertrophy and elevated LVSP and LVEDP, as previously described.[26-28] Although PKI-166 did not prevent LV hypertrophy, the drug completely normalized elevated LVEDP observed in 5/6Nx. An increase in LVEDP is an early sign for LV diastolic dysfunction[29] and has been shown to be an independent predictor of future clinical heart failure events[30] and mortality.[31-32] These results indicate that EGFR kinase inhibitors may have a therapeutic potential to limit the cardiac risks in patients with CKD.

Several experimental studies suggested EGFR inhibitors to possess renoprotective effects.[21-23] Of note is the contribution of Ang II in the development of renal fibrotic lesions.[20, 33-34] Recently, the renal fibrotic role of Ang II was linked to EGFR transactivation via AT_1 receptor-induced shedding of membrane-bound EGFR ligands through activation of ADAM (a disintegrin and metalloprotease).[35-36] In a study by Francois *et al.*, EGFR inhibition by gefitinib (also an inhibitor of EGFR tyrosine kinase) limited renal fibrosis, but did not display an antihypertensive action in nitric oxide deficiency-induced hypertension (L-NAME) model.[20] However, in contrast to this study in rats, gefitinib was shown to induce renal dysfunction in humans.[37-38] In our study, PKI-166 failed to improve renal injury, but prevented the increase in blood pressure. Therefore, the blood pressure lowering action mechanism of PKI-166 seems likely to be extrarenal. Previously, EGFR signaling was found affected by several antihypertensive therapies such as RAS inhibitors, endothelin 1 receptor antagonists and antioxidants[9, 39-41]. Moreover, the ACE inhibitor, imidapril,[39] AT_1 receptor antagonist, losartan,[42] and aldosterone antagonist, spironolactone,[40] reduce the

level of EGFR phosphorylation in different tissues. Our observation that PKI-166 treatment failed to exert renoprotection, but successfully lowered the blood pressure, fuels the idea that the above mentioned antihypertensive agents may decrease blood pressure, at least in part, by interfering with EGFR signaling.

The attenuated contractile response to Ang II and PE in the aorta of vehicle-treated 5/6Nx rats was successfully restored by either PKI-166 or lisinopril, which provides additional support for the extrarenal blood pressure lowering effect of PKI-166. In a previous study, partial nephrectomy was shown to cause a downregulation in vascular α1-AR[43] which was thought to be related with higher levels of circulating catecholamines after renal mass reduction.[43-44] Therefore, downregulation in vascular α1-AR may offer an explanation for the lower contractile responses to PE. In the SHR rat model, a gradual attenuation of maximal response to Ang II was shown starting from week 4 (non hypertensive) through week 16 (severe hypertension). Attenuated Ang II mediated aorta contractility was also shown in Obese Zucker Diabetic rat, an experimental model for genetic obesity with progressive renal injury.[45] Moreover, our analysis demonstrated a negative correlation between SBP and the maximal contraction response to Ang II or PE. Collectively these data suggest a causal role for hypertension in the attenuation of PE and Ang II response in aorta.

In conclusion, we investigated the therapeutic potential of EGFR inhibition initiated at an advanced disease stage in reno-cardiovascular disease. An important novel finding of this study is that the progression of hypertension was prevented by *in vivo* treatment with PKI-166, an EGFR kinase inhibitor. To the best of our knowledge, this is the very first *in vivo* study demonstrating CV protective effects of an EGFR inhibitor in CKD independent of modulation of renal injury. Our findings strongly suggest that the antihypertensive effect of EGFR inhibition is extrarenal, possibly by limiting transactivation of EGFR. Therefore, this study provides evidence for EGFR signaling as a target in CKD associated cardiovascular complications.

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Chapter 8

Summary and future perspectives

(submitted)

Introduction

In resistance arteries, myogenic constriction (MC) is activated by elevated transmural pressure, which causes contraction of arterial smooth muscle leading to reduction of vessel lumen thereby reducing blood flow in target organs. Myogenic constriction, as a vascular autoregulatory mechanism, helps to maintain stable blood flow over a wide systemic pressure range. In the kidney, increased intraglomerular pressure is a key determinant of the development of renal damage. Consequently, healthy glomeruli highly depend on the integrity of the preglomerular vasculature. Myogenic constriction of preglomerular arteries serves as a protective mechanism against large fluctuations in systemic pressure and as a mechanism to prevent elevated systemic pressure being transferred into the glomerulus. Aims of this thesis are:

3. to study MC to investigate whether:
    a. higher MC of preglomerular vessels is associated with lower vulnerability of kidneys to damage in several rat models: reduced renal mass/hypertension – 5/6Nx (**chapter 2**), type 2 diabetes mellitus – ZDF rat (**chapter 6**), and in ageing induced hypertension with predisposition to kidney damage – FH rat (**chapter 4**)
    b. MC of mesenteric arteries is affected by kidney disease with and without hypertension (**chapters 5, 6, 7)**
    c. pharmacological treatment of kidney disease with and without hypertension influences MC (**chapters 5, 6, 7**)
4. to identify whether *in vivo* glomerular vascular contractility to Angiotensin II predicts individual susceptibility to CKD in the adriamycine model and in 5/6Nx model, the latter also showing the relationship to MC (**chapter 2, 3**)

In this thesis, we show that higher MC of renal preglomerular vasculature is associated with lower vulnerability of the kidneys to damage in three rat models of chronic kidney disease (CKD), *i.e.*, 5/6Nx, the ZDF rat and the FH rat. Furthermore, we examined MC of mesenteric artery, as a representative of the peripheral vasculature, in these models and demonstrate that development of hypertension - but not CKD - is associated with decreased MC in this artery. Finally, we show that several types of treatment improve MC in both the renal and peripheral vascular bed of rats with CKD with and without hypertension.

The key features of the various models and the effects on the MC of renal and mesenteric arteries are summarized in Table 1 and discussed in the next paragraphs. Interestingly, the

pattern merging from this table suggests that from the clinical features (*i.e.* renal failure, hypertension), one may predict the change of MC in specific vascular beds.

In addition to the importance of MC as determinant of vascular smooth muscle function, we demonstrate that healthy rats with basal higher reactivity of glomerular arterioles to angiotensin II assessed *in vivo* developed higher renal damage in two distinct models of CKD.

The studies presented in this thesis therefore demonstrate that the patency of vascular smooth muscle contraction in small renal arteries is pivotal in limiting renal damage and limiting progression of renal disease.

Table 1) Summary of differences in myogenic constriction in mesenteric and renal arterial bed in different rat models of chronic kidney disease with or without hypertension. Y – indicates yes, N – indicates no, norm – indicates normal/physiologic, down – indicates decreased, weeks – indicates age in weeks, ARB – Angiotensin receptor blocker, EGFRB – EGF receptor blocker, rest of abbreviations is used in text. a *–Vavrinec et al., unpublished data*

	condition		myogenic constriction	
	HYPERTENSION	**RENAL FAILURE**	**MESENTERIC**	**RENAL**
FHH 12 weeks	N	Y	norm	down
FHL 12 weeks	N	N	norm	norm
FHH 52 weeks	Y	Y	down	down
FHL 52 weeks	Y	N	down	norm
ZDF	N	Y	norm	down
lean ZDF	N	N	norm	norm
5/6Nx	Y	Y	down	down [a]
5/6Nx + ARB	N	N	norm	norm [a]
5/6Nx + EGFRB	N	Y	norm	-

2. Renal myogenic constriction predicts susceptibility to renal damage

In **chapter 2**, we provide evidence that MC of renal interlobar arteries obtained from the extirpated kidney obtained at 5/6 nephrectomy (5/6Nx) predicts renal damage developing in the 12 weeks following the 5/6Nx. Consequently, animals with a lower MC at baseline, while still being healthy, subsequently developed increased renal damage compared to animals with higher MC at baseline. This finding fuels the assumption that patent (normal, healthy) MC of renal arteries protects the kidney from impairment of function and development of structural damage.

With increasing age, the incidence of hypertension rises, posing additional risk for impairment of renal function as well[1]. Therefore, in **chapter 4,** we studied ageing in a rat model of genetic predisposition for CKD – the Fawn Hooded rat. The key observation was that both 52 weeks old FHL and FHH show similarly increased blood pressure, but only FHH develops renal damage. Most likely, the absence of renal damage in FHL is due to preservation of MC of the renal vasculature in FHL, thus limiting glomerular hypertrophy and damage. Apart from the absence of renal damage, this is evidenced by the smaller glomerular cross-sectional area in FHL. The negative relation between MC and glomerular cross-sectional area was observed in both FH strains. This finding suggests that pharmacologic intervention maintaining renal MC is able to limit or prevent renal damage in hypertensive patients. This is of particular value in those patients in which hypertension is difficult to redress despite extensive therapy.

Taken the above together, we provide strong evidence from 2 rat models that patent MC is essential in prevention of renal damage related to the development of hypertension, either as a consequence of disease or related to increasing age.

3. Failure of renal myogenic constriction leads to renal damage, but not to hypertension

Patients with CKD are at higher risk of death due to a cardiovascular event rather than due to end-stage renal disease. Therefore, we wanted to investigate to what extend hypertension is associated with changes of renal MC and related renal failure. In three models with renal failure we explored MC, from which only two were also hypertensive. In all three models, renal failure developed and a decrease in renal MC was found. As ZDF rats were not hypertensive, it may be concluded that decline of MC constriction of preglomerular arteries is not associated with hypertension. Moreover, it is most likely involved in impaired renal autoregulation in the models[2-4] which causes renal damage regardless of hypertension.

In 5/6Nx, loss of MC is most likely driven by barotraumatic pressure overload due to presence of hypertension over the time of remnant glomerular arteries and arterioles following the surgical procedure. This subsequently leads to adaptation processes of vascular smooth muscle leading to loss of MC. In FHH rats, MC dysfunction seems to have a genetic background, as these rats are born with this dysfunction[5] prior to development of hypertension. The occurrence of hypertension early in life also has a genetic background in FHH, and several related loci have been identified[6] . The ZDF rats display impaired MC most likely due to the diabetes development, where oxidative stress causes several types of vascular dysfunctions[7]. Possibly, diabetes may cause impairment of renal MC as well, *e.g.*, also through increased oxidative stress (see below). Importantly, this model did not have hypertension, but displayed decreased renal MC indicating that impairment of renal preglomerular MC is not associated with the development of hypertension.

4. Failure of mesenteric myogenic constriction is associated with hypertension

Table 1. summarizes the results of MC with respect of localization of the arteries studied (renal or mesenteric) and the disease symptoms of animals. It clearly points out that the MC of peripheral mesenteric arteries is impaired in hypertensive animals, irrespective of the presence of renal failure. Thus, in contrast to systemic endothelial function[8-10], renal failure does not seem to affect MC of systemic arteries.

The general concept of essential hypertension is that increased peripheral resistance causes elevated blood pressure. Increased peripheral resistance can result from several mechanisms, including the increase in MC and arterial stiffening. Nevertheless, we observed that hypertensive 5/6Nx rats showed loss of MC in the peripheral (mesenteric) artery. This observation indicates that hypertension in this model is not caused by increased peripheral resistance. The 5/6Nx hypertensive model is different from essential hypertension in that it spontaneously occurs. Therefore, these findings are likely to be limited to renin-dependent hypertension. However, 52 weeks old FHL rats gradually developed hypertension during of aging and also showed loss of MC in the mesenteric arterial bed. Therefore, also this hypertensive model, which is closer to essential hypertension, the assumption that hypertension is caused by increased myogenic constriction of peripheral small resistance arteries seems not to hold true.

Arterial stiffening, the increase in rigidity of the peripheral vasculature, is considered another possible reason of increased peripheral resistance. In hypertension, the contractile phenotype of vascular smooth muscle cells is shifted towards the proliferative phenotype

with accumulation of extracellular matrix[11], impairing the ability of smooth muscle to constrict/relax. We have observed thicker arterial walls (although non-significant) in mesenteric arteries isolated from 5/6Nx rats, which is in line with the above mentioned shift in phenotype. Importantly, passive stretch curves of mesenteric arteries from 5/6Nx in response to stretch as obtained in calcium free physiological solution were similar to those of healthy rats or 5/6Nx rats treated with RAAS intervention. Moreover, in the aging FHL rat, no differences were observed between passive curves when compared to renal and mesenteric arteries of FHH, or with other rat strains used in this study. Therefore, an increase in peripheral vascular rigidity does not seem to play a role in the pathogenesis of hypertension in the models examined.

Moreover, our data suggest that loss of MC in mesenteric artery in hypertension is most likely an adaptation process to the increased blood pressure independent of kidney disease. Possibly, sustained activation of MC by the elevated pressure causes impaired MC. Therefore, we investigated whether in the 5/6Nx model hypertension or CKD causes loss of MC in mesenteric arteries using different treatments. We showed that the impaired MC in hypertensive animals was restored to the level of healthy sham operated animals after treatment with the angiotensin receptor blocker losartan (**Chapter 5**), the ACE-inhibitor lisinopril (**Chapter 7**) and an EGF-receptor blocker (**Chapter 7**). All of these treatments lowered blood pressure, but only losartan and lisinopril improved renal function. It therefore seems feasible that blood pressure reduction is enough to protect the mesenteric vascular bed from loss of MC. This observation also suggests that hypertension *per se* drives the loss of MC in mesenteric arteries in the models studied.

Finally, observations from our models seem to support the notion that hypertension is a prerequisite for loss of mesenteric MC. The ZDF rat with CKD, but without hypertension, had unchanged MC of mesenteric artery. Yet, the 52 weeks old FHL rat with hypertension but without CKD had decreased MC of the mesenteric artery. Taken the above together, these results support the theory that hypertension and not CKD drives loss of myogenic constriction in the mesenteric artery.

5. Possible causes of decreased MC in renal and mesenteric vascular bed

Involvement of reactive oxygen species of decreased MC

The impact of reactive oxygen species (ROS) on endothelial relaxation has been studied in detail, while the impact of ROS on myogenic constriction is less well explored. In the vascular smooth muscle, Angiotensin II activates NADPH oxidases leading to formation of

superoxides[12]. In pathological conditions, when superoxides are overproduced, these react with NO reducing its bioavailability, while generating peroxynitrite ($ONOO^-$). Importantly, it was described that peroxynitrite inhibits *in vitro* myogenic response of rat posterior cerebral artery via protein nitrosilation and subsequent depolymerization of F-actin. Moreover, peroxynitrite was also found in cerebral arteries in cerebral ischemia reperfusion injury, where decreased MC was explained by the peroxynitrite associated decrease of F-actin[13;14] . In addition, we recently found peroxynitrite to inhibit MC in renal arteries as well (Vavrinec *et al.*, unpublished). Therefore, we propose that ROS are involved in impaired renal preglomerular MC of ZDF and therefore impaired renal autoregulation. We found normalized expression of renal tissue p22phox and restored renal MC in vildagliptin treated ZDF, (**chapter 6**). Here, p22phox, a subunit of NOX-2, is localized in smooth muscle of renal vessels. Therefore, treatment with vildagliptin of ZDF reducing the expression of p22phox may have led to lower production of superoxide and subsequent decreased formation of peroxynitrite and preservation of MC. Furthermore, we found that *in vitro* incubation of mesenteric arteries with ROS scavengers partially reverse the decline of MC in 5/6Nx rats (**chapter 5**). This again suggests that ROS plays a role in lowered MC of small resistance arteries.

Myogenic constriction is impaired due to overall decreased contractility of vascular smooth muscle

One of the possible reasons for the decreased MC may be a generally lowered ability of arterial smooth muscle to constrict. Once the contractile apparatus of arterial smooth muscle cells is impaired, MC is inhibited as well. Nevertheless, we observed normal or even increased agonist induced contractility of arteries (mesenteric or renal) in which MC was impaired. In ZDF rats, which display decreased MC in the renal interlobar artery, we observed increased responsiveness of renal arteries to the alpha-adrenoceptor agonist phenylephrine and normal responses to depolarizing agents, such as potassium chloride (**chapter 6**). Therefore, we conclude that decreased MC was not caused by an overall impairment of contractility of arterial smooth muscle. Additionally, we found a strong positive correlation between PE mediated constriction and MC of renal arteries isolated from healthy rats (Figure 1; Vavrinec *et al.*, unpublished). Consequently, it seems likely that the mechanism causing the decline in MC is located upstream of phospholipase C (PLC) – perhaps at level of the sensor of the pressure changes or the MC specific calcium influx mechanism. Unfortunately, this part of the signal transduction route that initiates MC is still incompletely explored.

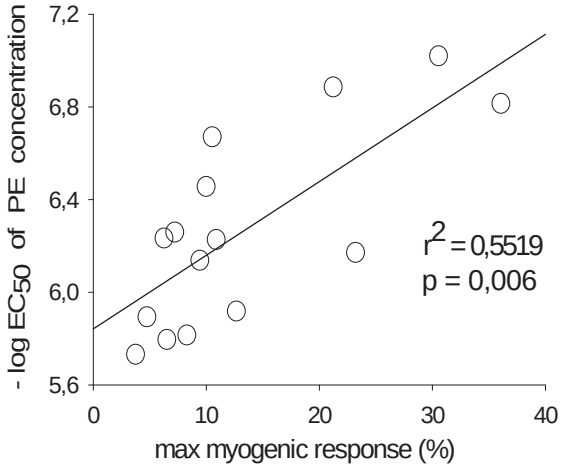

Figure 1. The relationship between phenylephrine (PE) mediated constriction and MC of renal interlobar arteries freed out of endothelium isolated from healthy 12 weeks old Wistar rat.

6. Predictive value of glomerular vasculature function to renal failure susceptibility

While the susceptibility to develop renal damage varies considerably among individuals, its determinants are still incompletely understood. The extent of existing systemic factors, such as diabetes or hypertension, cannot fully explain the predisposition to renal failure [15], suggesting additional factors to be involved. In general, the factors governing this individual vulnerability to renal damage are thought to be intrinsic to the kidney and probably largely genetically determined [2].

Previous results from our lab suggest that renal vascular function (endothelium mediated relaxation) assessed under healthy conditions can predict the susceptibility to renal failure in *e.g.* 5/6Nx, in cardio-renal interaction (myocardial infarction combined with unilateral nephrectomy) or in adriamycin-induced nephrosis. These facts highlight the importance of endothelial function, while in this thesis the importance of vascular smooth muscle function as predictor of vulnerability to CKD is underscored. We showed that MC of renal arteries of healthy rat can predict subsequent damage after 5/6Nx and contractility of afferent and efferent arterioles to angiotensin II as well.

Using intravital microscopy that allows visualization of the glomerulus and glomerular arterioles, we showed that rats with a higher response to angiotensin II assessed prior to the 5/6Nx surgical procedure developed increased renal damage 12 weeks thereafter (**chapter 2**). Moreover, a negative correlation was found between MC of interlobar arteries and

angiotensin II evoked responses of glomerular arterioles. This relationship indicates how well balanced the contractile properties of vascular smooth muscle over the intrarenal vascular bed are needed for healthy kidney. Moreover, in the adriamycin model, healthy arteriolar reactivity to angiotensin II *in vivo* assessed prior to disease induction predicts the severity of CKD (**chapter 3**). Thus, from these experiments we can conclude that measurement of the response of glomerular arteries may serve as basis to develop diagnostic tools to identify patients with higher susceptibility to renal damage. Moreover, we confirmed the role of angiotensin II in pathogenesis of renal damage.

Angiotensin II binds to several receptors. The most pronounced effect of angiotensin II is mediated via Angiotensin type 1 receptor. Angiotensin type 1 receptor (AT1R) has been described also as a stretch sensor in the cell. We have previously shown interaction between AT1R and MC in a model of heart failure, where increased MC was restored by an inverse agonist of the AT1R. Decreased number of caveolae in vascular smooth muscle in this model or pharmacological disruption of caveolae is associated with increased MC of small arteries. Therefore, we can hypothesize that possible role of AT1R in MC generation or AT1R as effectors of Ang II could be dependent upon the location in the membrane - in or outside caveolae. Unfortunately, there is limited additional data and further research is needed to substantiate this hypothesis.

7. Conclusion and future perspectives

Endothelial function has long been implied in the pathogenesis of cardiovascular and renal diseases and hypertension. Consequently, research of vascular function in these conditions is focused more towards endothelium than vascular smooth muscle, even though vascular smooth muscle contractility is the main regulatory mechanism of small arterioles influencing peripheral resistance. In this thesis we therefore investigated the importance of contractility of arterial smooth muscle, *i.e.*, myogenic constriction and reactivity to angiotensin II in renal and peripheral (mesenteric) arteries and whether these functions can predict the outcome of chronic kidney disease. We showed that myogenic constriction of preglomerular arteries (assessed *in vitro*) and angiotensin II mediated constriction of glomerular arterioles (*in vivo*) assessed prior to induction of the disease can predict the renal outcome of CKD.

Taken together, our studies imply that vascular contractility of renal vessels is a highly important mechanism involved in the pathogenesis of CKD. Effectively, these studies

suggest vascular smooth muscle to represent an important target for therapy and diagnostics.

The exact intracellular mechanism of myogenic constriction is still incompletely understood, especially the trigger of this mechanism is so far poorly known. Therefore, treatment interventions that target myogenic constriction are still lacking. Nevertheless, we showed improved myogenic constriction of renal and mesenteric arteries of rats with CKD and/or hypertension 1. after treatment with RAAS inhibition, which improved renal function and lowered blood pressure, 2. GLP-1 signaling where improvement of renal MC might have led to reduction in glomerulosclerosis, and 3. intervention with epidermal growth factor signaling, that had no effect on kidney damage, but lowered blood pressure, thus improving MC of mesenteric artery. This suggests that therapies that indirectly support maintenance of MC can improve/restore MC as well. However, additional characterization of mechanisms of vascular smooth muscle contractility, in particular those involved in MC, and the relationship with pathogenic factors will be needed to disclose innovative means to prevent and treat chronic kidney disease.

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Samenvatting

Introductie

In vaten die een belangrijke rol spelen in bloeddrukregulatie, de zogenaamde weerstandsvaten, wordt de myogene constrictie geactiveerd door verhoogde transmurale druk. De verhoogde transmurale druk zorgt voor contractie van het arteriële gladde spierweefsel, met vernauwing van het lumen en daardoor verminderde bloeddoorstroming naar de organen als gevolg. Myogene constrictie is een belangrijk autoregulatoir mechanisme van vaten: het draagt bij aan een constante doorbloeding van organen ondanks variaties in systemische bloeddruk. In de nier is een toename van de intraglomerulaire druk een belangrijke factor in de ontwikkeling van nierschade. Voor het goed functioneren van de glomeruli is de integriteit van de preglomerulaire vaten van cruciaal belang. Myogene constrictie in de preglomerulaire arteriën beschermt de glomeruli tegen grote fluctuaties in systemische bloeddruk.

In dit proefschrift laten we zien dat een hogere myogene constrictie van renale preglomerulaire vaten, geassocieerd is met een verminderde kwetsbaarheid voor nierschade in drie ratmodellen van chronische nierziekten: de 5/6 nefrectomie, de Zucker Diabetic Fatty (ZDF) rat en de Fawn Hooded rat. Verder hebben we in deze modellen de myogene constrictie onderzocht in mesenterische vaten, als maat voor de perifere vasculaire autoregulatie. Hierin lieten we zien dat de ontwikkeling van hypertensie, maar niet die van chronische nierziekten, geassocieerd is met een verminderde myogene constrictie in deze arteriën. Tenslotte hebben we laten zien dat verschillende behandelingen myogene constrictie in zowel renale als perifere vaten van ratten met chronische nierziekten, met en zonder hypertensie, kan verbeteren.

In **hoofdstuk 2** laten we zien dat myogene constrictie in interlobulaire arteriën van gezonde nieren verkregen na een 5/6 nefrectomie, de ontwikkeling van nierschade 12 weken na de nefrectomie in de andere nier kan voorspellen. Hoe lager de myogene constrictie van interlobulaire arteriën in de gezonde rat, hoe groter de ontwikkeling van nierschade ten opzichte van ratten met een hoge basale myogene constrictie. Deze onderzoeksresultaten ondersteunen de aanname dat een goed functionerende myogene constrictie van renale arteriën de nieren beschermt tegen functieverlies en de ontwikkeling van structurele schade.

Vergrijzing en myogene constrictie

Met het toenemen van de leeftijd neemt ook de incidentie van hypertensie toe. Dit vormt een bijkomend risico voor het verslechteren van de nierfunctie. Daarom hebben we in **hoofdstuk 4** het effect van 'ageing'onderzocht in een rat model met een genetische predispositie voor het ontwikkelen van chronische nierziekte: de Fawn Hooded rat. De belangrijkste observatie was dat zowel 52 weken oude FHL en FHH ratten een gelijke toename in bloeddruk vertonen, maar dat alleen de FHH ratten nierschade ontwikkelen. De afwezigheid van renale schade in FHL ratten is waarschijnlijk te danken aan de preservatie van myogene constrictie in renale arteriën in deze ratten, waardoor er minder glomerulaire hypertrofie en nierschade ontstaat. Naast de afwezigheid van nierschade, wordt dit ondersteund door een verminderd glomerulair cross-sectioneel gebied in FHL ratten. De negatieve relatie tussen myogene constrictie en het glomerulaire cross-sectioneel gebied werd waargenomen in zowel FHH als FHL ratten. Deze bevindingen suggereren dat farmacologische interventie, gericht op het handhaven van de myogene constrictie, gebruikt kan worden voor de preventie en vermindering van nierschade in patienten met hypertensie. Dit is van extra belang in patienten die slecht reageren op bloeddrukreducerende medicatie. Bovenstaande samenvattend, tonen we in 2 ratmodellen aan dat een goed functionerende myogene constrictie essentieel is voor de preventie van nierschade gerelateerd aan de ontwikkeling van hypertensie, als consequentie van ziekte of gerelateerd aan toenemende leeftijd.

Het falen van renale myogene constrictie leidt tot nierschade, maar niet tot hypertensie

Patiënten met chronische nierziekten hebben een groter risico op overlijden door hart- en vaatziekten dan door het bereiken van het eindstadium van de nierziekte zelf. Daarom hebben we onderzocht in welke mate hypertensie is geassocieerd met verandering in myogene constrictie en daaraan gerelateerd nierfalen. We hebben myogene constrictie bestudeerd in 3 modellen van nierfalen, waarvan 2 met hypertensie. In alle drie de modellen trad er nierschade op, waarbij een vermindering van de renale myogene constrictie werd gevonden. Aangezien de ZDF ratten niet hypertensief waren, kunnen we concluderen dat de achteruitgang in myogene constrictie van preglomerulaire arteriën niet geassocieerd is met hypertensie. Achteruitgang in myogene constrictie is waarschijnlijk dus betrokken bij de verslechterde renale autoregulatie in deze modellen, onafhankelijk van het optreden van hypertensie.

Veroorzaken van verminderde myogene constrictie

Gevolg van verminderde myogene constrictie in verschillende vasculaire gebieden zijn nog niet volledig begrepen, hoewel verscheidene hypothesen die dit fenomeen te beschrijven is gepostuleerd. In de 5/6 Nx ontstaat het verlies van myogene constrictie het meest waarschijnlijk door een teveel aan barotraumatische druk in glomerulaire arteriën en arteriolen door het postoperatieve optreden van hypertensie. Dit leidt vervolgens tot adaptieve processen van het vasculaire gladde spierweefsel waardoor er een verlies van de myogene constrictie ontstaat. In FHH ratten lijkt het optreden van disfunctie van de myogene constrictie een genetische achtergrond te hebben, aangezien bij deze ratten de disfunctie al aanwezig is voordat ze hypertensie ontwikkelen. Bij de ZDF rat treedt de disfunctie in myogene constrictie het meest waarschijnlijk op door de ontwikkeling van diabetes, waarbij oxidatieve stress verschillende types vasculaire disfunctie kan veroorzaken. Mogelijk zorgt ook diabetes via toegenomen oxidatieve stess voor een verslechterd functioneren van de myogene constrictie. Ondanks dat in dit model geen hypertensie optreedt, was er een afname van de myogene constrictie. Dit geeft aan dat de verslechtering van renale preglomerulaire myogene constrictie niet geassocieerd is met de ontwikkeling van hypertensie.

Vermindering van MC in mesenteriale vaten wordt veroorzaakt door hoge bloeddruk en niet nierfalen

We hebben laten zien dat de verslechterde myogene constrictie in hypertensieve dieren werd hersteld tot op het niveau van controle ratten na behandeling met de angiotensine receptor blokker Losartan (**hoofdstuk 5**), de ACE-remmer Lisinopril (**hoofdstuk 7**) en een EGF-receptor blokker (**hoofdstuk 7**). Al deze behandelingen verlaagden de bloeddruk, maar alleen losartan en lisinopril verbeterden de renale functie. Het verlagen van de bloeddruk lijkt daarom afdoende om het mesenteriale vasculaire vaatbed te beschermen tegen een verlies van myogene consrictie. Deze observatie suggereert ook dat hypertensie op zichzelf de drijvende kracht is achter het verlies van myogene constrictie in mesenteriale vaten in de bestudeerde modellen. Tot slot lijken observaties in onze modellen de aanname te ondersteunen, dat hypertensie een voorwaarde is voor het verlies van mesenteriale myogene constrictie. In de ZDF rat, een model waarbij chronisch nierfalen optreedt zonder hypertensie, was er geen verandering in myogene constrictie in mesenteriale arteriën. 52 week oude FHL ratten met hypertensie maar zonder chronisch nierfalen, hadden daarentegen een verlechterde myogene constrictie in de mesenteriale arteriën.

Bovenstaande waarnemingen ondersteunen de theorie dat hypertensie, en niet chronisch nierfalen, leidt tot verlies van myogene constrictie in mesenteriale arteriën.

Glomerulaire vasculaire functie als voorspellende waarde voor het ontwikkelen van nierfalen

De gevoeligheid voor het optreden van nierschade varieert sterk tussen individuen en de determinanten zijn nog onvoldoende bekend. De mate van bestaande systemische factoren, zoals diabetes of hypertensie, kunnen de predispositie voor het ontstaan van nierfalen onvoldoende verklaren, wat suggereert dat additionele factoren betrokken zijn. Er wordt verondersteld dat deze factoren die de individuele gevoeligheid voor nierschade verklaren lokale intrarenale factoren zijn die grotendeels genetisch zijn bepaald.

Voorafgaand onderzoek in ons lab suggereert dat renal vasculaire functie (endotheel gemedieerde relaxatie), gemeten onder gezonde omstandigheden, de gevoeligheid voor nierfalen voorspelt na 5/6Nx, na cardio-renale interactie (myocard infarct gecombineerd met unilaterale nefrectomie) en na adriamycine geïnduceerde nefrose. Dit onderzoek richtte zich op het belang van de endotheelfunctie. In dit proefschrift hebben we het belang van het vasculaire gladde spierweefsel als voorspeller voor de gevoeligheid voor het ontstaan van chronisch nierfalen onderzocht. We hebben laten zien dat myogene constrictie van renale arteriën van gezonde ratten, de nierschade na 5/6Nx en contractiliteit van afferente en efferente artriolen op angiotensine II kan voorspellen.

Door het gebruik van intravitale microscopie konden we de glomerulus en de glomerulaire arteriolen in vivo bestuderen. We hebben laten zien dat ratten met een sterkere respons op angiotensine II voorafgaand aan een 5/6 Nx operatie, in grotere mate nierschade ontwikkelen 12 weken na 5/6Nx (**hoofdstuk 2**). Verder werd er een negatieve relatie gevonden tussen myogene constrictie van interlobulaire arteriën en angiotensine II geïnduceerde responsen van glomerulaire arteriolen. Deze relatie geeft aan hoe belangrijk de balans van contractiele eigenschappen van het vasculaire gladde spierweefsel in het intrarenale vasculaire bed nodig is voor een gezonde nierfunctie. Daarnaast hebben we in het adriamycine model laten zien dat gezonde arteriolaire reactivieit op angiotensine II in vivo, bepaald voor de inductie van ziekte, de mate van het optreden van chronische nierziekte voorspelt (**hoofdstuk 3**). We kunnen dus concluderen dat het meten van de respons van glomerulaire arteriën als basis kan dienen voor het ontwikkelen van diagnostische testen om patiënten met een hogere gevoeligheid voor nierschade te

identificeren. Bovendien hebben we de rol van angiotensine II in de pathogenese van nierschade bevestigd.

Conclusie en toekomstperspectief

Endotheeldisfunctie wordt al sinds lange tijd als een belangrijke factor gezien in de pathogenese van hart- en vaatvaatziekten, nierfalen en hypertensie. Hierdoor richt veel onderzoek naar vaatfunctie zich meer op endotheelfunctie dan op het functioneren van het vasculaire gladde spierweefsel, terwijl de contractiliteit van het spierweefsel van kleine arteriolen het belangrijkste regulatoire mechanisme is voor de perifere weerstand. In dit proefschrift hebben we daarom het belang van de contractiliteit van het arteriële gladde spierweefsel onderzocht. Hierbij hebben we gekeken naar myogene constrictie en reactiviteit van de vaten op angiotensin II in renale en perifere (mesenterische) arteriën. Bovendien hebben we gekeken of deze vaatfunctie het ontstaan van chronische nierziekte kan voorspellen.

Samenvattend laten onze studies zien dat veranderde vasculaire contractiliteit van renale vaten een belangrijk mechnisme is in de pathogenese van chronische nierziekte. Deze studies suggereren dat het vasculaire gladde spierweefsel een belangrijk aangrijpingspunt vormt voor therapie en diagnostiek.

Er is nog veel onduidelijk over het exacte intracellulaire mechanisme van myogene constrictie, met name over de triggers voor dit mechanime is weinig bekend. Hierdoor zijn er nog weinig therapieën die zich richten op het verbeteren van de myogene constrictie. Desalniettemin heben we een verbetering van myogene constrictie gezien van renale en mesenterische arteriën van ratten met chronische nierziekte en/of hypertensie 1. Na RAAS remming, dat de bloeddruk verlaagde en de renale functie verbeterde. 2. Na interventie in de GLP-1 signaaltransductie, waar de verbetering van renale myogene constrictie heeft geleid tot reductie van glomerulosclerose en 3. na Interventie in de EGF signaaltransductie, dat geen effect had op nierschade, maar de bloeddruk verlaagde en de myogene constrictie in de mesenteriale arteriën verbeterde. Dit geeft aan dat therapieën die indirect het behoud van de myogene constrictie ondersteunen, de myogene constrictie mogelijk ook kunnen verbeteren/ herstellen. Desondanks zal verdere kennis over de mechanismes van de contractiliteit van het vasculaire gladde spierweefsel nodig zijn, met name over de mechanismes die betrokken zijn bij myogene constrictie en de relatie tot pathogene factoren. Op deze manier kunnen nieuwe methodes ontwikkeld worden om nierschade te voorkomen en te behandelen.

Zhrnutie a záver

Úvod

Myogénna konstrikcia (MC) je v rezistentných artériach aktivovaná zvýšeným transmurálnym tlakom, ktorý vedie ku kontrakcii hladkého svalu artérie, čo spôsobuje redukciu lúmenu cievy a tým aj redukciu toku krvi v cieľovom orgáne. Ako cievny autoregulačný mechanizmus napomáha udržiavať stabilný prietok krvi cez široký rozsah systémového krvného tlaku.

Pretože zdravie glomerulov závisi aj od integrity preglomerulárnej vaskulatúry, kľúčovým determinantom rozvoja renálneho poškodenia je zvýšený intraglomerulárny tlak. Myogénna konstrikcia preglomerulárnych artérií slúži aj ako protektívny mechanizmus proti veľkým fluktuáciam v systémovom krvnom tlaku a tiež zabraňuje prenosu zvýšeného tlaku krvi do glomerulov.

V predkladanej dizertačnej práci sme dokázali, že vyššia MC renálnej preglomerulárnej vaskulatúry je spojená so zníženou náchylnosťou obličky na poškodenie, na čo sme použili tri experimentálne modely chronickej choroby obličiek (chronic kidney disease- CKD), a to: 5/6 nefrektómiu (5/6Nx, chirurgická ablácia 5/6-tín oboch obličiek), Zucker Diabetic Fatty rat (ZDF- potkany s diabetom druhého typu) a Fawn Hooded rat (FH potkany, s genetickou predispozíciou pre CKD a hypertenziu). Ďalej sme sledovali MC mezenterálnej artérie, ktorá reprezentuje periférnu vaskulatúru a ukázali sme, že vývoj hypertenzie (ale nie CKD) je spojený so zníženou MC v tejto artérii. Na záver sme sledovali vplyv rôznych druhov terapie na MC v renálnej i periférnej vaskulatúre potkanov s CKD v prítomnosti a neprítomnosti hypertenzie.

V **kapitole 2** ukazujeme**,** že MC renálnych intralobárnych artérií, izolovaných z obličky potkana tesne po 5/6Nx, dokáže predpovedať rozsah renálneho poškodenia, vyvinutého 12 týždňov po operácii. Znamená to, že zvieratá s nižšou bazálnou MC vyvinuli väčšie renálne poškodenie v porovnaní so zvieratami, ktorých bazálna MC bola zvýšená. Podporili sme teda hypotézu, že patentná - normálna, čiže zdravá MC renálnych artérií ochraňuje obličku pred zhoršením funkcie a v konečnom dôsledku pred vývojom poškodenia.

Starnutie a myogénna konstrikcia

Keďže so zvyšujúcim sa vekom narastá výskyt hypertenzie, a tým aj riziko renálneho poškodenia, v **kapitole 4** sme sa zamerali na skúmanie vplivu procesu starnutia na MC v modele genetickej predispozície pre CKD (FH rat). Kľúčovým poznaním bolo, že 52 týždňov staré FHH potkany (H-hypertensive; hypertenzia v 12 týždni veku) aj FHL (L-low

blood pressure; nízky tlak krvi v 12 týždni veku) mali rovnako zvýšený tlak, ale iba FHH vyvinuli renálne poškodenie. FHL potkany boli ochranené pred poškodením pravdepodobne kvôli zachovanej MC preglomerulárnych artérií, čo limitovalo glomerulárnu hypertrofiu a poškodenie. Dôkazom bola aj menšia veľkosť glomerulov u tohto podkmeňa podkanov. Túto hypotézu potvrdzuje aj negatívna korelácia medzi veľkosťou glomerulov a MC u oboch podkmeňov. Náš poznatok by mohol položiť základy pre farmakologickú intervenciu, ktorá by bola schopná zachovať MC a tým limitovať/predísť renálnemu poškodeniu u starších hypertenzných pacientov. Zhrnúc horeuvedené fakty sme dospeli k záveru, že patentná MC je esenciálna v prevencii renálneho poškodenia v prítomnosti hypertenzie.

Zlyhanie renálnej myogénnej konstrikcie nevedie k hypertenzii, ale ku poškodeniu obličky

Pretože kardiovaskulárne príhody u pacientov s CKD prispievajú väčšou mierou k riziku úmrtia, než samotné konečné štádium obličkového zlyhania, naším ďalším cieľom bolo zistiť, do akej miery je hypertenzia spojená so zmenami renálnej MC, a tým aj s poškodením samotnej obličky. MC sme skúmali v troch potkaních modeloch renálneho zlyhanie, z čoho len dva boli hypertenzné. Vo všetkých modeloch však bolo zistené zlyhanie obličiek a MC. Pretože ZDF potkany hypertenzné neboli, môžeme tvrdiť, že znížená MC preglomerulárnych artérií nie je spojená s hypertenziou. Navyše znížená MC zjavne viedla k vzniku CKD v daných modeloch.

Dôvody zníženej MC

I keď niekoľko hypotéz, objasňujúcich zníženie myogénnej konstrikcie v rôznych vaskulárnych oblastiach už bolo postulovaných, tento fenomén doteraz nebol uspokojujúco objasnený. Strata MC pri 5/6Nx je pravdepodobne spôsobená barotraumatickým preťažením artérii tlakom krvi z dôvodu hypertenzie, čo vedie k adaptačným mechanizmom v hladkej svalovine cievy, vedúc následne k strate MC. Pri FHH potkanoch je dysfukcia MC podmienená genetickým základom, pretože zvieratá vyvinú túto dysfunkciu pred tým, ako sa u nich hypertenzia prejaví. U ZDF potkanov diabetes spôsobuje zvýšený výskyt voľných radikálov, čo vedie k vaskulárnym dysfunkciám rôzneho typu a teda aj k strate MC.

Zníženie MC mezenterálnych artérií je spôsobené hypertenziou a nie renálnym zlyhaním

Ukazali sme, že patologicky znížená MC *mezenterálnych* artérií u hypertenzných potkanov bola obnovená po liečbe: losartanom – blokátorom angiotenzínoveho receptora (**kapitola 5**), lizinoprilom – ACE inhibítorom (**kapitola 7**) a blokátorom EGF receptora (**kapitola 7**). Všetky tieto terapie viedli k zníženiu krvného tlaku, avšak iba losartan a lisinopril zlepšili zhoršenú funkciu poškodených obličiek. Preto sa zdá byť zjavné, že redukcia tlaku krvi je dostatočnou ochranou mezenterálnych artérií pred stratou myogénnej funkcie. Z tohto pozorovania vyplýva, že nie obličkové zlyhanie, ale hypertenzia *per se* vedie k strate MC v mezenterálnych artériách.

I keď ZDF potkany mali plne rozvinutú CKD (avšak bez prítomnosti hypertenzie) zmenu MC v mezenterálnych artériách sme nezaznamenali. Avšak u 52 týždňových hypertenzných FHL potkanov bez CKD sme zníženú MC mezenterálnych artérií pozorovali. To znamená a ďalej podporuje teóriu, že strata MC v periférnych - mezenterálnych artériách je spôsobená nie CKD, ale naopak hypertenziou.

Prediktívna hodnota funkcie glomerularnej vaskulatúry pre náchylnosť k obličkovému zlyhaniu

Náchylnosť k obličkovému zlyhaniu je v populácií veľmi variabilná a jej determinanty nie sú stále úplne objasnené. Faktory ako diabetes, či hypertenzia nevysvetľujú dostatočne predispozíciu pre CKD. To naznačuje existenciu ďalších faktorov, ktoré musia byť v predispozícií pre CKD zahrnuté. Vo všeobecnosti sa predpokladá, že faktory zodpovedné za individuálnu predispozíciu k CKD sú intrinzitné obličke a do veľkej miery determinované geneticky.

Naše predošlé pozorovania poukazujú na to, že renálna vaskulárna funkcia (endotelom mediovaná relaxácia) meraná v zdravom stave, dokáže predpovedať náchylnosť na obličkové zlyhanie, a to v modeloch ako 5/6Nx, v modele kardio-renálnej interakcie (indukovaný infarkt myokardu v kombinácii s unilaterálnou nefrektómiu), alebo v modele adriamycínom indukovanej CKD. Tieto poznatky vyzdvyhujú dôležitosť funkcie endotelu. V predkladanej práci sme však sústredili pozornosť na funkciu hladkého svalu artérií ako prediktora náchylnosti k CKD. Podarilo sa nám dokázať, že MC renálnych intralobárnych artérií a reaktivita aferentnej a eferentnej arterioly na angiotenzín II (Ang II), stanovená v zdravom štádiu, dokáže predpovedať renálne poškodenie v 5/6Nx.

Za použitia intravitálnej mikroskopie, ktorá umožňuje vizualizáciu glomerulárnych arteriol *in vivo*, sme ukázali, že potkany s bazálne vyššou odpoveďou na Ang II, stanovenou pred 5/6 Nx, následne vyvinuli 12 týždňov po zákroku väčšie poškodenie (**kapitola 2**). Nadôvažok sme pozorovali negatívnu koreláciu medzi MC intralobárnych artérií a Ang II vyvolanou konstrikciou glomerulárnych ciev. Tento vzťah poukazuje na vyváženosť kontraktilných vlastností hladkej svaloviny renálnej vaskulatúry, čiže na zdravé fungovanie obličky. Ďalej sme v adriamycínovom modeli ukázali, že reaktivita glomerulárnych artérií na Ang II, stanovená pred podaním adriamycínu, dokáže predikovať rozsah renálneho poškodenia, podobne ako pri 5/6 Nx (**kapitola 3**). Na základe týchto experimentov môžeme teda uzavrieť, že stanovenie funkcie glomerulárnych arteriol by mohlo slúžiť ako základ pre vývoj diagnostických pomôcok, ktoré by pomohli identifikovať pacientov s vyššou náchylnosťou na vznik obličkového poškodenia, a to dávno pred rozvinutím samotného ochorenia.

Záver a perspektívy do budúcnosti

Funkcia endotelu je už dlho implikovaná v patogenéze kardiovaskulárnych a renálnych ochorení i hypertenzie. V dôsledku toho je výskum funkcie ciev väčmi zamerý na endotel, ako na hladkú svalovinu ciev, i keď kontraktilita hladkého svalstva artérií je hlavným regulačným mechanizmom malých arteriol, ovplyvňujúcich periférnu rezistenciu. V našej práci sme sa preto zamerali na význam kontraktility hladkej svaloviny artérií a to myogénnu konstrikciu a reaktivitu na angiotenzín II v renálnych a periférnych (mezenterálnych) artériách a taktiež na schopnosť týchto funkcií predvídať rozsah chronického ochorenia obličiek. Ukázali sme, že myogénna konstrikcia preglomerulárnych artérií (*in vitro)* a angiotenzínom II sprostredkovaná konstrikcia glomerulárnych arteriol (*in vivo*), merané pred vyvolaním ochorenia obličiek, dokáže predvídať výsledok CKD.
Celkovo vzaté, naše štúdie naznačujú, že vaskulárna kontraktilita renálnych artérií je mechanizmus nevyhnutne sa podieľajúci na patogenéze CKD a že hladká svalovina ciev predstavuje dôležitý cieľ pre diagnostiku a následnú terapiu.

Presný intracelulárny mechanizmus myogénnej konstrikcie nie je doposiaľ celkom objasnený a to najmä jeho spúšťače. Liečebné intervencie, ktoré sa zameriavajú na ovpyvnenie myogénnej konstrikcie, preto stále chýbajú. Napriek tomu sme zaznamenali zlepšenie myogénnej konstrikcie renálnych a mezenterálnych artérií u potkanov s chronickým obličkovým ochorením alebo hypertenziou: 1. po liečbe RAAS inhibítormi, ktoré zlepšili funkciu obličiek a znížili krvný tlak; 2. GLP-1 intervenciou, kde zlepšenie

renálnej MC viedlo k zníženiu glomerulosklerózy a 3. zásahom do signalizácie epidermálneho rastového faktoru (EGFR), ktorý znížil krvný tlak a tým zlepšil myogénnu konstrikciu mezenterálnych artérií. To znamená, že aj terapie, ktoré nepriamo zasahujú do mechanizmu myogénnej konstrikcie, môžu viesť k jej samotnému zlepšeniu. Na rozvinutie inovatívnych prístupov pre prevenciu, diagnostiku a liečbu chronických ochorení obličiek, je však nevyhnutná ďaľšia charakteristika mechanizmov kontraktility hladkej svaloviny artérií a to najmä zložiek, podieľajúcich sa na myogénnej konstrikcii i na jej vzťah s patogénnymi faktormi.

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